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REGULATION OF CONNECTIVE TISSUE GROWTH FACTOR/CCN2 GENE EXPRESSION IN SYSTEMIC SCLEROSIS FIBROBLASTS

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**This report is submitted in fulfillment of the
requirements for the degree of PhD in the
University of London**

2006

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Abstract

Systemic sclerosis (Scleroderma, SSc) is a chronic, connective tissue disease of unknown etiology, characterised by vascular dysfunction, inflammation and organ fibrosis. Involving both genetic and environmental components, the specific mechanisms which result in fibrosis remain largely unknown. A cardinal feature of SSc is increased synthesis of extracellular matrix (ECM). Dermal fibroblasts cultured from SSc patients maintain many of the abnormal properties seen *in vivo*, including excess production of collagen type I, and growth factors such as connective tissue growth factor (CTGF/CCN2). CTGF, like many genes dysregulated in SSc, is induced by TGF- β in normal fibroblasts. The overall aim of my studies was to determine the mechanism(s) controlling CTGF over-expression in SSc dermal fibroblasts (SDF).

Induction of CTGF by TGF- β was found to be dependent upon elements in the proximal portion of the CTGF promoter, distinct from those of the previously characterised TGF- β response element (TRE). The TRE acts, in NIH/3T3 and HFF cells, as a regulator of basal expression, and is not essential for TGF- β induction of CTGF. Instead TGF- β induces CTGF expression via a Smad3 complex, binding to a *bona fide* SMAD transcription factor binding site. Over-expression CTGF in SDF is independent of autocrine expression of TGF- β and the SMAD binding element and rather dependent on a functional Sp-binding site. Inhibition of Sp1-like DNA binding reduces excessive CTGF expression in SDF. Consistent with this Sp1-DNA binding activity is elevated in SDF nuclear extracts. Investigation of the mechanism of elevated Sp1-like binding found that SDF exhibited constitutively active ERK1/2 and JNK1. Inhibition of ERK1/2 repressed elevated Sp-binding and CTGF over-expression observed in SDF.

In summary, the data presented in this thesis provide evidence that dysregulation of ERK1/2 in SDF is involved in CTGF over-expression via a Sp1-like DNA binding. Thus repression of ERK may represent a candidate in targeting fibrosis in SSc.

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List of Abbreviations

ACA	Anti-centromere antibodies
ACE	Angiotensin-converting enzyme
APS	Ammonium persulphate
ATA	Anti-topoisomerase antibodies
α-SMA	alpha-smooth muscle actin
α-MEM	α -minimal essential medium
μl	Microlitres, volume
AB	Antibody
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
BMP	Bone morphogenic protein
°C	Degrees Celsius, temperature
CO₂	Carbon dioxide
Col-1	Collagen, type 1
CM	Conditioned media
Co-Smad	Common mediator Smad
CTGF	Connective tissue growth factor
DcSSc	Diffuse cutaneous systemic sclerosis
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethylsulphoxide
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbant assay
ET-1	Endothelin-1
FCM	Fibroblast conditioned medium
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FGM	Fibroblast growth medium
FN	Fibronectin
H1	Hour, time
GVHD	Graft versus host disease
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
ICAM	Intercellular adhesion molecule
IGF	Insulin-like growth factor 1
IPAH	Isolated PAH
I-Smad	Inhibitory Smad
IL-1	Interleukin-1
kd	Kilodaltons, weight
KGF	Keratinocyte growth factor
LAP	Latency-associated peptide
LcSSc	Limited cutaneous systemic sclerosis
LTBP	Latent TGF- β -binding protein
M	Molar
min	Minute, time
ml	Millilitre, volume
MMP	Matrix metalloproteinase

mRNA	Messenger RNA
ng	Nanogram, weight
PAH	Pulmonary arterial hypertension
PI3-kinase	Phosphatidylinositol-3-OH kinase
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
PBS-T	PBS-Tween 20
PDGF	Platelet derived growth factor
PMSF	Phenylmethylsulphonylfluoride
RP	Raynaud's phenomenon
RNA	Ribonucleic acid
RNP	Ribonuclear protein
ROS	Reactive oxygen species
rpm	Revolutions per minute, speed
R-Smad	Receptor-regulated Smad
SARA	Smad Anchor for Receptor Activation
SD	Standard Deviation
SDF	SSc dermal fibroblasts
SDS	Sodium dodecylsulphate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
sec	second, time
Smad	A merger of Sma from <i>Caenorhabditis elegans</i> and Mad from <i>Drosophila mothers against decapentaplegic</i>
SNP	Single nucleotide polymorphism
SRC	Scleroderma renal crisis
SSc	Systemic sclerosis
TBS	Tris buffered saline
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF-α	Transforming growth factor- alpha
TGF-β	Transforming growth factor-beta
TIMP	Tissue inhibitor of matrix metalloproteinases
TNF-α	Tumour necrosis factor-alpha
tPA	Tissue-type plasminogen activator
Tsk1/+	Tight skin mouse
uPA	Urokinase-type plasminogen activator
V	Voltage, power
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor

Papers:

1. Shi-wen,X., Pennington,D., **Holmes,A.**, Leask,A., Bradham,D., Beauchamp,J.R., Fonseca,C., du Bois,R.M., Martin,G.R., Black,C.M., and Abraham,D.J. (2000). Autocrine over-expression of CTGF maintains fibrosis: RDA analysis of fibrosis genes in systemic sclerosis. *Exp. Cell Res.* 259, 213-224.
2. **Holmes,A.**, Abraham,D.J., Sa,S., Shiwen,X., Black,C.M., and Leask,A. (2001). CTGF and SMADs, maintenance of scleroderma phenotype is independent of SMAD signaling. *J. Biol. Chem.* 276, 10594-10601.
3. Leask,A., Sa,S., **Holmes,A.**, Shiwen,X., Black,C.M., and Abraham,D.J. (2001). The control of *ccn2* (ctgf) gene expression in normal and scleroderma fibroblasts. *Mol. Pathol.* 54, 180-183.
4. Stratton,R., Shiwen,X., Martini,G., **Holmes,A.**, Leask,A., Haberberger,T., Martin,G.R., Black,C.M., and Abraham,D. (2001). Iloprost suppresses connective tissue growth factor production in fibroblasts and in the skin of scleroderma patients. *J. Clin. Invest* 108, 241-250.
5. Leask,A., **Holmes,A.**, and Abraham,D.J. (2002). Connective tissue growth factor: a new and important player in the pathogenesis of fibrosis. *Curr. Rheumatol. Rep.* 4, 136-142.
6. **Holmes,A.**, Abraham,D.J., Chen,Y., Denton,C., Shi-wen,X., Black,C.M., and Leask,A. (2003). Constitutive connective tissue growth factor expression in scleroderma fibroblasts is dependent on Sp1. *J. Biol. Chem.* 278, 41728-41733.
7. Leask,A., **Holmes,A.**, Black,C.M., and Abraham,D.J. (2003). Connective tissue growth factor gene regulation. Requirements for its induction by transforming growth factor-beta 2 in fibroblasts. *J. Biol. Chem.* 278, 13008-13015.
8. Xu,S.W., Howat,S.L., Renzoni,E.A., **Holmes,A.**, Pearson,J.D., Dashwood,M.R., Bou-Gharios,G., Denton,C.P., du Bois,R.M., Black,C.M., Leask,A., and Abraham,D.J. (2004). Endothelin-1 induces expression of matrix-associated genes in lung fibroblasts through MEK/ERK. *J. Biol. Chem.* 279, 23098-23103.
9. Shi-Wen X, Rodriguez-Pascual F, Lamas S, **Holmes A**, Howat S, Pearson JD, Dashwood MR, du Bois RM, Denton CP, Black CM, Abraham DJ, Leask A. (2006). Constitutive ALK5-independent c-Jun N-terminal kinase activation contributes to endothelin-1 overexpression in pulmonary fibrosis: evidence of an autocrine endothelin loop operating through the endothelin A and B receptors. *Mol Cell Biol.* 26:5518-27.
10. **Holmes, A.**, and Abraham,D.J. (2006). New Insights into the Role and Regulation of CTGF/CCN2 in Fibrotic Disorders: Submitted- *Am J Path*
11. **Holmes, A.**, Shiwen, X., Ponticos M, Abraham, D.J. (2006). Differential expression of the CCN family members Cyr61, CTGF and Nov in fibrosis. In preparation

12. **Holmes, A., Shiwen, X., Leask A, Lindyl G, Abraham, D.J. (2006). Serum induction of CCN2 is ERK/Sp dependent: Role in maintaining fibrogenesis. In preparation**
13. **Holmes, A., Shiwen, X., Abraham, D.J. Differential regulation of CCN2 promoter activity by AP-1 family members. In preparation**

Declaration

I declare that all the experiments in this thesis were carried out by myself, unless otherwise stated:

The work described in Chapter 4; Section 4.4.2, entitled **Response of SSc fibroblasts to TGF- β stimulation**, was performed in collaboration with Dr Sarah Howart, Kings College, London. Complimentary cDNA of the samples were made by myself and Q-PCR was performed by Dr Howart (Kings College London). CTGF promoter deletion and point mutation constructs were generated in collaboration with Dr Andrew Leask (University of Western Ontario).

Professor David Abraham PhD
Supervisor

Acknowledgements

First I am of course indebted to my supervisors, Professors David Abraham and Carol Black and Dr George Martin, who have given me the opportunity to work in such a fascinating and fulfilling area of medical research. In particular I wish to thank David for his support and encouragement over the years. The whole of the department of Rheumatology deserves a mention for providing such an enjoyable work environment. In particular Drs Gisela Lindahl, Markella Ponticos, Vin Rajkumar and Xu Shi-wen who have debated and shared the many trials and tribulations of this research and my 'roomie' Dr Carmen Fonseca, my sincere thanks. To Dr Andrew Leask for his infectious and ceaseless enthusiasm for CTGF and Professor Jeremy Pearson, Dr Charlie Waters and Dr Hynda Kleinman who have offered invaluable help and advice over the course of the PhD. Finally I wish to pay tribute to Dr Anita Roberts' generosity to a lowly PhD student.

I have been in the enviable position of having a supportive family and group of friends. First to my family: to my parents Mavis and Ron, and Auntie Dorothy, my nephew Robert and my outlawed sister, Janet, my thanks. To a collection of friends who still have the ability to surprise me with the depth of their kindnesses. To Steve and Kath, Dave and Bec, and my much loved godchildren, my thanks for ensuring my feet stayed firmly on the ground. To Paul, for his sage like over-view of medicine and good company and my long suffering friends in London, William for his patience for my miss use of the, comma, Lynsay, Billy, Scott, Eram, Anil, Howie and Mary who have kept me sane over this time, and Richard, 'Mel', my long suffering house mate, who has made the latter part of the PhD coffee and cake filled at all possible points- my deepest thanks.

'I dedicate this thesis in the memory of a much missed brother, James, whom I wish I had the opportunity of knowing better'

Chapter 1

Introduction

1.1 Overview

Fibrosis may be defined as the excessive accumulation of scar tissue which leads to the distortion of normal tissue architecture (Krieg and LeRoy, 1998; LeRoy, 1985). Under normal circumstances the extracellular matrix (ECM), which constitutes scar tissue, is tightly regulated by the resident cells. Upon tissue injury, such as wounding, a highly orchestrated sequence of events involving many different cell types, and soluble mediators, including cytokines and growth factors, leads to the replacement of the lost and damaged tissue as efficiently and effectively as possible. By contrast to this ordered repair process, fibrosis results from the uncontrolled accumulation of ECM. Fibrosis is a pathological hallmark of many human diseases, including pulmonary fibrosis, hepatic cirrhosis, keloids and chronic glomerulonephritis. A prototypic example of a fibrotic disorder is systemic sclerosis (SSc), in which the majority of tissues may be affected. The pathogenesis of SSc is uncertain but believed to involve both genetic and environmental components. The disease is characterised by immune activation, vascular damage and ultimately replacement fibrosis. Central to the development of tissue fibrosis is the dysregulated expression of components of the ECM and growth factors by the SSc fibroblast. With no proven therapies able to arrest or prevent fibrotic diseases, understanding the molecular mechanisms responsible for the expression of these pro-fibrogenic genes under normal and pathological situations is likely to provide valuable insight into the development and maintenance of fibrosis. The studies described in this thesis will focus on a growth factor over-expressed by SSc dermal fibroblasts, connective tissue growth factor (CTGF/CCN2), and the normal and pathological mechanisms underlying its cellular expression. This introductory chapter will give an overview of the current understanding of SSc and the underlying cells and mechanisms key to the development of fibrosis. Further I will discuss the CCN family of genes, of which CTGF represents a prototypic member, focusing on the current understanding of CTGF regulation and function.

1.2 Systemic Sclerosis

Hippocrates (460-377 BC) was the first to describe a skin disorder comparable to SSc. However the first convincing description of the disease appeared in a monograph written by Carlo Curzio in Naples in 1753 (Barnett, 1996). SSc was first given the name 'sclérodermie' by Gintrac in 1847. However it was not until 1924 when Matsui described five patients with sclerodermie involving the gastrointestinal tract, kidneys and lungs, that the visceral involvement was recognised. Subsequently papers by Goetz and others have led to the adoption of the current name, 'Systemic Sclerosis', with 'scleroderma' (LeRoy, 1985; Barnett, 1996) referring to a spectrum of disorders outlined in Table 1.1.

SSc is a heterogeneous rheumatological connective tissue disorder of unknown aetiology (Denton et al., 2006). Although uncommon it has a significant mortality, largely as a result of connective tissue fibrosis. The disease is grouped into subsets based upon clinical and serological criteria (Table 1.2). SSc is characterised by the accumulation of extracellular matrix, resulting in connective tissue fibrosis. The most evident signs of SSc are manifested in the skin and it is to this that the disorder owes its original name, scleroderma (Greek: *skleros*, hard, and *derma*, skin). Pathologically SSc is divided into several processes: (1) microvascular injury; (2) inflammation and the development of autoantibodies; (3) accumulation of ECM and tissue fibrosis; (4) atrophy. Tissue fibrosis is almost universally preceded by microvascular dysfunction and inflammation in the development of the disease, suggesting these phases represent a early stages in the disease process (Black, 1995). In SSc patients virtually all organ systems can be affected by fibrosis to various degrees; however of major clinical significance are the heart, gastrointestinal tract, kidneys and lungs.

Disease	Subtype
<i>I- Raynaud's phenomenon</i>	Raynaud's disease (primary) Raynaud's syndrome (secondary)
<i>II- Scleroderma</i>	
1) Systemic	Limited Cutaneous systemic sclerosis (lcSSc) Diffuse Cutaneous systemic sclerosis (dcSSc) Scleroderma sine scleroderma
2) Localised	Morphea Linear En coup de sabre
3) Juvenile	Localised forms Systemic forms
4) Chemically induced	Environmental/occupational Drugs
<i>III- Scleroderma-like diseases</i>	Metabolic Immunological/inflammatory Eosinophilic fasciitis Eosinomyalgic syndrome Mixed connective tissue disease (MCTD) Overlap syndromes

Table 1.1
Scleroderma spectrum of conditions.
 Modified from Black *et al.* (Black and Denton, 1998b)

1.2.1 Epidemiology

SSc has a worldwide distribution affecting both sexes. Incidence rates, from retrospective reviews, vary from 2-19 cases per million population per year, and the prevalence has been reported between 19 and 75 per 100,000 (Maricq et al., 1989). SSc is gender biased with females 3 to 8 times more likely to develop the disease. The peak incidence of the disease in the general population is during the fourth and sixth decades, although it can occur earlier and during childhood (Silman and Newman, 1996). The overall survival rate of SSc patients is 60-83% at 5 years and 40-75% at 10 years (Ioannidis et al., 2005; Nietert et al., 2005), however this varies between disease subsets. Currently the major cause of mortality within the diffuse disease subset is renal crisis in the first five years of onset and pulmonary complications (Steen and Medsger, Jr., 2000). Pulmonary arterial hypertension (PAH) represents the leading cause of mortality in patients with the limited cutaneous disease (Mukerjee et al., 2003). The Choctaw Indians, a native North American tribe, have an increased prevalence of scleroderma (469 per 100,000 population). Choctaw scleroderma patients have a particularly uniform diffuse disease with prominent lung involvement and anti-topoisomerase I autoantibodies (Arnett 1996).

1.2.2 Classification criteria for SSc and subgroups

The 1980 Systemic Sclerosis (SSc) classification criteria were proposed by a committee of the American Rheumatism Association. For the purpose of classifying patients in clinical trials, population studies and research, clinical features of SSc are defined into major and minor clinical criteria (Appendix I). More recently an amendment was proposed in order to include the increasing number of patients that have SSc characteristics but do not fulfil these criteria. This early or 'pre-SSc' subgroup has a vascular defect called Raynaud's Phenomenon (RP), serological marker (at least one SSc-specific autoantibody) and an SSc pattern of nailfold capillaries, but lack fibrosis (LeRoy et al., 1988).

The most commonly adopted classification for SSc subgroups (Leroy et al., 1988) is based upon the extent of clinical involvement of the skin, serological markers and natural history associations. Clinically affected areas of skin are often referred to as involved, and regions clinically unaffected on the same patient as uninvolved. The classification based on skin involvement essentially divides the systemic disease into limited cutaneous systemic sclerosis (lcSSc) and diffuse cutaneous systemic sclerosis

(dcSSc). For an overview of the current classification see Table 1.2. Over 60% of the patients are classified as lcSSc, where the involvement of internal organs tends to occur late in the evolution of the disease and is usually preceded by RP, often for many years. The onset of the dcSSc tends to be more rapid, with organ failure often present within the first 5 years of the disease (Black and Denton, 1998a).

1.2.3 Aetiology

Whilst the aetiology of SSc remains unknown, there is evidence supporting both environmental and genetic contributions. Collective studies support the role of genetics in disease development and progression. Although, SSc is not inherited in a Mendelian manner, studies by Arnett *et al.* have shown first-degree relatives of SSc patients to have an increased risk (less than 1%), albeit slight, of developing the disease (Arnett *et al.*, 2001). In addition, the low disease concordance in monozygotic twins (Zhou *et al.*, 2005b) implies a genetic pre-disposition that requires a secondary trigger, such as environmental, for SSc to fully develop.

Interestingly a high prevalence and homogenous presentation of SSc has been reported in a Native American tribe, the Choctaw Indians. Genome-wide association studies have led to the identification of several specific genetic loci which are associated with susceptibility for SSc (Zhou *et al.*, 2003). One such locus maps to a region containing the fibrillin-1 gene (Tan *et al.*, 1998). A spontaneous duplication in this gene has been described in the murine type I tight skin (Tsk1/+) model of SSc (See section 1.3). The use of candidate gene approaches and functional genomics has identified a number of genetic associations. Several single nucleotide polymorphisms (SNPs) associated with SSc have been identified in a number of genes including: (1) Chemokines such as monocyte chemoattractant protein-1 (MCP-1); (2) Growth factors, including transforming growth factor-beta (TGF- β); (3) Extracellular matrix proteins such as fibronectin and fibrillin, and matricellular proteins, including SPARC (Tan *et al.*, 2001; Ahmed and Tan, 2003).

Subset	Characteristics
1. "Pre-scleroderma"	<ul style="list-style-type: none"> • Raynaud's phenomenon plus nailfold capillary changes • Digital ischaemic changes • Disease specific circulating antibodies (ATA/ACA)
2. Diffuse cutaneous SSc (dcSSc)	<ul style="list-style-type: none"> • Onset of skin changes within 1 year of onset of Raynaud's. • Truncal or acral involvement. • Presence of tendon friction rubs. • Early and significant incidences of interstitial lung disease, oliguric renal failure, diffuse gastrointestinal disease and myocardial involvement. • Nailfold capillary dilatation and drop out. • ATA in ~30% of patients.
3. Limited cutaneous SSc (lcSSc)	<ul style="list-style-type: none"> • Raynaud's phenomenon for several years • Skin sclerosis restricted to extremities, face and neck. • Significant involvement of pulmonary hypertension, skin calcification, telangiectasiae and gastrointestinal involvement. • High incidence of ACA (70-80%)
4. Scleroderma sine scleroderma	<ul style="list-style-type: none"> • With or without Raynaud's phenomenon • No skin involvement. • Pulmonary fibrosis, scleroderma renal crisis, cardiac or gastrointestinal disease. • Antinuclear antibodies may be present.

Table 1.2

Systemic Sclerosis subsets classification.

Anti-centromere antibodies (ACA); Anti-topoisomerase I autoantibody (ATA).

Modified from Black *et al.* (Black and Denton, 1998b)

Several studies have demonstrated modest associations of the major histocompatibility complex, human leukocyte antigen (HLA) alleles and SSc. For example, weak association with HLA-DRB1*08 is reported with African American SSc patients, whereas HLA DR5 (DRB1*1101 and *1104, DQA*0501, DQB1*0301) and DR3 haplotypes (DRB1*0301, DQA1*0501, DQB1*0301) are more commonly associated with American and European Caucasians (Johnson et al., 2002). By contrast specific HLA alleles are strongly associated with autoantibody subsets. HLA-DRB1*11 and HLA-DPB1*1301 correlate with the presence of anti-topoisomerase I autoantibody (ATA) while HLA-DRB1*01, DRB1*04, and DQB1*0501 are associated with anti-centromere antibodies (ACA) (Gilchrist et al., 2001; Reveille et al., 2001). Thus, rather than affecting susceptibility to SSc, it is likely HLA genes influence specific immune responses in patients.

Several environmental factors have been proposed in SSc pathology, including infectious agents such as viruses (Lunardi et al., 2005), and oxidative stress leading to free radical damage (Svegliati et al., 2005; Herrick and Matucci, 2001). In addition exposure to silica has been associated with the development of SSc particularly in coal miners and stonemasons (Silver, 1996a). A number of other chemical agents have been put forward as causative agents, including epoxy resins, toxic oil in addition to organic solvents such as vinyl chloride, but the evidence remains inconclusive (Nietert and Silver, 2000). Of note has been the proposal that the exchange of cells at birth between mother and child leads to human leukocyte antigen (HLA) class II incompatibility and produces a graft-versus-host reaction, seen in animal models of the disease (see section 1.3), clinically leading to SSc (Nelson et al., 1998). Termed the microchimerism hypothesis, it may offer a plausible explanation for the female propensity of the disease, however this remains to be fully determined (Jimenez and Artlett, 2005).

1.2.4 Clinical and serological features of SSc

SSc is a heterogeneous disease with patients exhibiting a wide spectrum of clinical and serological characteristics.

1.2.4.1 Clinical characteristics

Raynaud's phenomenon

The episodic vasoconstriction of small arteries and arterioles of the extremities is defined as Raynaud's phenomenon (RP). A hallmark of the disease, RP affects between 90-95% of SSc patients, and is often their first symptom of the underlying SSc disease. These vasospasm events may be brought on by cold exposure, vibration or emotional stress, and may precede skin sclerosis by several years (Wigley, 1996). Population studies suggest that RP is a common disorder with prevalence of RP in the US ranging from 2-6% (Suter et al., 2005). Less than 5% of all people with RP may progress to develop the connective tissue disease (Wigley et al., 1992a; Belch, 1991; Suter et al., 2005).

Skin Sclerosis

Almost all SSc patients have skin sclerosis, but the extent and severity of this differ between patients, reflecting the heterogeneous nature of this disease. Skin sclerosis is used as a measure of disease activity (Black and Denton, 1998b; Sato et al., 2000), and the extent of skin involvement is the single major criterion for sub-classification of SSc into the principle subsets- lcSSc and dcSSc (Black and Denton, 1998b). Thus it is widely accepted that patients with sclerosis of the skin proximal to the neck, elbows and knees have dcSSc, but those with involvement distal to with distal involvement to these are designated as having lcSSc. In addition to differences in the extent and severity of skin involvement between the subsets, changes over time in the same patient are observed. Skin involvement in SSc usually progresses in three phases; oedematous, indurative and finally atrophic (Figure 1.1). The skin changes usually begin distally in the extremities with the advancing proximal edge referred to as the leading edge. The early oedematous phase is marked by swelling underneath the skin of the fingers, hands and other extremities including the face and is associated with inflammation. Subsequently the skin gradually becomes firm and thickens (indurative phase) and is referred to as involved skin. Regions that appear normal on patients are referred to as unaffected, although several studies suggest this region and the resident cells within are in fact sub-clinically abnormal (Claman et al., 1991). In patients with the dcSSc, skin

changes become generalised, including all the extremities, face, trunk and abdomen. Rapid progression of these changes over a relatively short period of time is usually associated with internal organ affection, especially lungs, kidneys and heart and these features are linked to a worse prognosis (Clements et al., 2000). The skin changes in dcSSc usually peak around 3-5 years and then slowly improve. In the localised subset, the skin events tend to be more gradual, and they are restricted to fingers, distal extremities and face. After many years the skin may soften and return to the normal thickness or become thin and atrophic (Black and Denton, 1998).

Gastrointestinal

Gastrointestinal manifestations are the most common symptoms in SSc patients, affecting 90% of patients. Symptoms include oesophageal, small-bowel and colon alterations. In addition to difficulty in swallowing, dysphagia and heartburn are frequent complaints, secondary to oesophageal hypo-motility. Small-bowel hypo-motility can lead to diarrhoea, weight loss and malabsorption (Black and Denton, 1998).

Renal disease

SSc renal crisis (SRC) was the major cause of mortality in SSc before effective therapies, such as angiotensin-converting enzyme (ACE) inhibitors, were introduced (Denton and Black, 2005). The use of ACE inhibitors has been associated with a change in survival rates in SRC patients from 10% to 70% at 5 years. Predominantly affecting dcSSc patients (12% dcSSc versus 2% lcSSc), SRC is associated with the presence of anti-RNA polymerase antibody (Bunn et al., 1998; Steen and Medsger, Jr., 2000).

Cardiac disease

SSc heart involvement may be either primary or secondary to lung/renal involvement. Clinically affecting 10% of all SSc cases, the two principle mechanisms involved are fibrosis as a result of myocardial RP or an immune-mediated myocarditis (Black and Denton, 1998b).

Macrovascular disease

In addition to the unifying feature of RP, which is microvascular disease, recent reports have found macrovascular involvement. Studies by Ho *et al.* have shown a significant increase in the prevalence of carotid artery disease in SSc patients compared to healthy controls, in addition to evidence of increased prevalence of peripheral arterial disease (Ho *et al.*, 2000).

Lung disease

Lung disease affects approximately 30% of all SSc patients, and in the advent of effective treatment of renal involvement and crisis, is now the primary cause of mortality (Steen *et al.*, 1994). The two major types of pulmonary disease affecting SSc patients are fibrosing alveolitis (FASSc) and pulmonary arterial hypertension (PAH) (Silver, 1996b).

FASSc is characterised by early alveolar inflammation and ultimately lung fibrosis and the histological pattern of fibrosis within the lung defines two distinct groups. The majority of SSc patients develop non-specific interstitial pneumonia pattern (NSIP) which is characterised by wide spread inflammation of the lung, whereas usual interstitial pneumonia (UIP) is defined by localised regions of lung involvement with fibrotic foci (Bouros *et al.*, 2002). FASSc is more frequent and severe in patients with dcSSc and with patients positive for ATA (Diot *et al.*, 1999).

PAH affects approximately 12% of patients with SSc and is defined by the development of pulmonary artery pressure higher than 30mmHg at rest (Mukerjee *et al.*, 2003). PAH can develop in dcSSc and lcSSc patients and is classified into two forms, primary or isolated PAH (IPAH) and secondary PAH. The development of secondary PAH is preceded by and is believed to result from the development of significant pulmonary fibrosis. IPAH is frequently associated with patients with lcSSc, and the presence of ACA and clinically undetectable pulmonary fibrosis (Coughlan and Mukerjee, 2001). The development of PAH in isolation or in association with pulmonary fibrosis is a major cause of mortality (MacGregor *et al.*, 2001).

1.1.4.3 Serological characteristics

Autoantibodies

Auto-antibodies against nuclear antigens (ANA), have been described in up to 95% of the patients with SSc (Bosworth *et al.*, 1998; Ho and Arthritis, 2003). There are 3 main patterns of ANA and a number of other relatively common serologic subgroups in SSc (Table 1.1). The main autoantibody specificities are closely associated with distinctive clinical profiles. For example, the AKA is more or less exclusively associated with

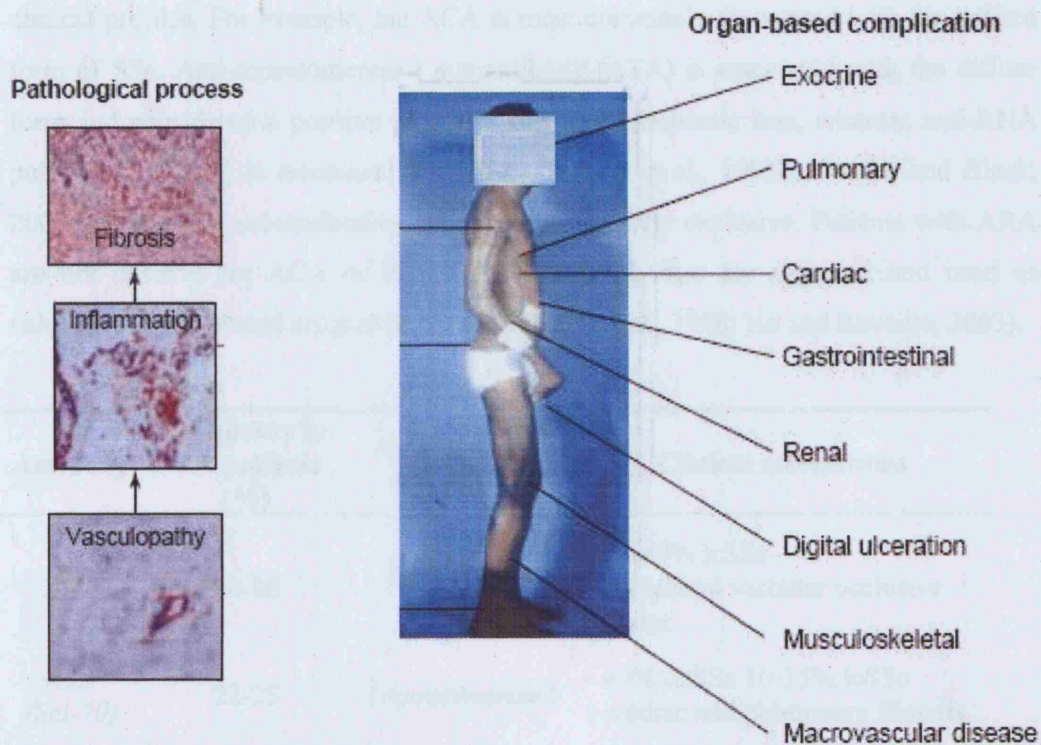


Figure 1.1

Differential organ involvement in SSc.

Pathologically SSc develops in four separate but linked phases. The earliest pathological feature of SSc is vascular defects in the skin (vasculopathy). Inflammation may occur simultaneously to vascular defects or subsequently follow. In turn these phases are replaced by fibrosis (left panels) and ultimately atrophy. SSc is a systemic disease (right panel) and similar processes which occur in the skin are likely to occur in all organs. Figure reproduced with authors' permission. Denton *et al.* (Denton and Black, 2005)

1.2.4.2 Serological characteristics

Autoantibodies

Antibodies against nuclear antigens (ANA) have been described in up to 95% of the patients with SSc (Bunn et al., 1998; Ho and Reveille, 2003). There are 3 main autoantibodies and a number of minor mutually exclusive serologic subgroups in SSc (Table 1.3). The major autoantibody specificities are usually associated with distinctive clinical profiles. For example, the ACA is most commonly associated with the limited form of SSc. Anti-topoisomerase I autoantibody (ATA) is associated with the diffuse form and considered a positive predictor for digital ischemic loss, whereas anti-RNA polymerase (ARA) is associated with SRC (Wigley et al., 1992b; Denton and Black, 2005). In addition autoantibodies appear to be mutually exclusive. Patients with ARA are not positive for ACA or ATA. Thus autoantibodies are regarded and used as valuable diagnostic and prognostic markers (Bunn et al., 1998; Ho and Reveille, 2003).

Antibody	Frequency in SSc patients (%)	Auto-antigens recognised	Clinical associations
<i>ACA</i>	20-26	Centromere proteins	- 70-80% lcSSc - Peripheral vascular occlusive disease
<i>ATA</i> (<i>Scl-70</i>)	22-25	Topoisomerase I	- 40% dcSSc 10-15% lcSSc - cardiac and pulmonary fibrosis
<i>ARA</i>	18-23	RNA polymerase	- 23 % dcSSc - Renal crisis
<i>UI-RNP</i>	10	U1 snRNP	- renal, PAH
<i>Anti-Pm-Scl</i>	4	Pm-Scl	- Scleroderma overlap - Polymyositis.
<i>Th/To</i>	4	40 kD Th ribonucleo protein	- Poor outcome in lcSSc
<i>AFA</i>	4-6	U3 RNP	- PAH and renal crisis - Afro-Caribbean population

Table 1.3

The Main serologic groups in SSc.

Anticentromere CENPs -A, -B, -C; RNA polymerase I, II, III. Modified from (Denton and Black, 2005; Black and Denton, 1998b).

1.2.5 The Pathogenesis of SSc

Whilst the aetiology of SSc remains largely unknown, the pathological interplay of vascular damage and immune activation is believed to promote fibroblast activation and increased synthesis and deposition of ECM proteins that culminate in tissue fibrosis (Figure 1.1).

1.2.5.1 Vascular damage

The vascular changes and damage in SSc are systemic affecting both micro and macro vessels. Endothelial damage is among the earliest events reported in the skin of SSc patients (Prescott et al., 1992). SSc microvessels are 'leaky' and result in the enhanced migration of inflammatory cells across the endothelial cell layer and into the extravascular space (Black and Denton, 1998b). In fibrotic human skin apoptotic endothelial cells have been detected in the inflammatory stage of disease (Sgonc et al., 2000). Anti-endothelial cell antibodies, present in the serum of SSc patients, induce endothelial cell apoptosis *in vitro* and may represent a link between immunological dysfunction and vascular damage (Distler et al., 2004; Sgonc et al., 2000). The microvascular pericyte is an integral part of the microvessel (Leveen et al., 1994). Elegant studies by Rajkumar *et al.* have demonstrated these cells are activated in the disease state and may directly contribute to tissue fibrosis through expression of ECM proteins and thus represent a link between microvascular damage and fibrosis (Rajkumar et al., 2005).

1.2.5.2 Immune activation

The presence of antinuclear antibodies, inflammatory lesions in the skin and increased profibrotic chemokines in the blood and tissues are features of the immune activation and inflammatory nature of the early stages of SSc (Black and Denton, 1998b; Zhou et al., 2005a; Querfeld et al., 1999). Increased numbers of immune cells, predominantly T cells, but also mast cells, eosinophils and basophils are found in an activated state in the tissues of SSc patients (Artlett, 2005). These cells are capable of modifying endothelial and fibroblast cell functions through the release of soluble mediators, such as TGF- β . Increasingly autoantibodies appear not only to be serological markers, but also mediators of the disease. Serum antibodies reacting with fibroblast plasma membrane antigens in SSc have been reported to be able to act as an extrinsic stimulus of fibroblast activation *in vitro*, inducing the expression of intracellular adhesion molecule 1 (ICAM-1) (Chizzolini et al., 2002). Autoantibodies against matrix proteases may contribute to

the development of fibrosis by inhibiting their activity in reducing the ECM (Sato et al., 2003). A recent report demonstrated that autoantibodies can promote the development of a 'fibrogenic' phenotype in normal fibroblasts. In this study Zhou *et al.* showed affinity-purified autoantibodies against portions of fibrillin-1, isolated from SSc sera, induced transcriptional expression of ECM genes, including several types of collagen and genes involved in matrix turnover, such as tissue inhibitor of metalloproteinases-1 (TIMP1) (Zhou et al., 2005a). The mechanisms by which these autoantibodies induce these gene changes is unclear, but potentially they may cause the release of sequestered TGF- β from fibrillin-1-containing microfibrils in the ECM.

1.2.5.3 Fibrosis

A unifying feature of SSc is increased synthesis and deposition of ECM macromolecules, predominantly fibrillar collagens. The ECM is composed of a wide variety of molecules and includes members of the collagen family, elastic fibers, glycosaminoglycans (GAG), proteoglycans, and adhesive glycoproteins (Mauch et al., 1993; Mutsaers et al., 1997). This connective tissue framework surrounds and supports tissues. The ECM was initially thought to act as an inert scaffold; however it is apparent the matrix can influence the cells in contact with it. The ECM affects cellular functions including migration, proliferation, differentiation (Pizzo et al., 2005) and in addition serves as a local reservoir of growth factors, such as TGF- β (Taipale et al., 1994) and members of the CCN family of proteins (Kireeva et al., 1997). The different combinations of these secreted substances that comprise the ECM give rise to various types of scaffolds that characterise the different body tissues and organs influencing the function of the resident cell types. Histological studies of SSc patients have shown that skin from affected areas exhibits a thickened dermis and a loss of the normal tissue architecture due to increased deposition of ECM (Figure 1.1). Patients with early scleroderma exhibit increased amounts of type III collagen deposits in the lower dermis whereas type I collagen production dominates in later stages of the disease (Fleischmajer et al., 1978; Prockop and Kivirikko, 1995). Subsequent studies have confirmed that the production of several connective tissue components (e.g. V, VI, and XVI collagens, decorin, proteoglycans, fibronectin and the ED-A splice variant of fibronectin) is also elevated in the dermis of SSc patients (Hesselstrand et al., 2002; Akagi et al., 1999; Rudnicka et al., 1994; Rajkumar et al., 2005; Kuroda and Shinkai, 1997). The elevated expression of ECM components and immune activation of SSc is reminiscent of processes which occur as a result of injury. Indeed, pathologically

fibrosis may arise from an inability to terminate a normal wound healing process (Leask and Abraham, 2004).

1.2.6 Wound repair

Physiologically, skin fibroblasts *in vivo* normally synthesise little ECM, such as collagen type I. However, a change in the balance in favour of the production of ECM is key in the replacement of tissue during wound healing and can, under pathological circumstances, lead to excessive ECM production and fibrosis (Ponticos et al., 2004; Schiller et al., 2004). Wound healing is tightly regulated and leads to the partial restoration of the structure and function of the damaged tissue efficiently and effectively. Although wound healing has been predominantly studied within the context of the skin parallel processes occur in the majority of tissues and involve three overlapping phases: inflammation; tissue formation; and tissue remodelling. The repair process is regulated by a network of growth factors and cytokines (Table 1.4). Wound healing is initiated immediately after injury by the release of growth factors and cytokines from the serum and degranulating platelets (Kubota et al., 2004; Liu et al., 2003; Singer and Clark, 1999). The formation of a blood clot comprised of cross-linked fibrin and ECM proteins including fibronectin, vitronectin and thrombospondin, acts both as a barrier to the external environment and as a scaffold for the migration of cells into the wound area (Singer and Clark, 1999). Responding to the released growth factors including TGF- β and platelet derived growth factor (PDGF), immune cells are first to enter the wound site and remove microorganisms and cell debris. The wound healing process is perpetuated by the subsequent release by invading macrophages of growth factors and cytokines including epidermal growth factor (EGF) which promotes the migration and proliferation of keratinocytes and TGF- β which promotes proliferation, migration and synthesis of new ECM by dermal fibroblasts (Singer and Clark, 1999). During this phase several cell types, including fibroblasts, pericytes and fibrocytes differentiate into myofibroblasts, and contract the wound via cellular adhesion to the collagen and fibronectin rich matrix (Singer and Clark, 1999; Abe et al., 2001). Following contraction cellular apoptosis of endothelial cells and myofibroblasts leads to an acellular collagen-rich scar (Clark, 1996). The cumulative effects of the loss of pro-fibrotic stimuli and changes in matrix turnover, regulated through the actions of proteolytic enzymes, matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) which inhibit MMP activity (Visse and Nagase, 2003; Brew et al., 2000) leads to scar resolution and termination of the wound healing

response. Unlike embryonic wound healing which leads to a scarless replacement of tissue (Martin, 1997), adult wound healing does not restore normal tissue structure but generates a satisfactory restoration of tissue which lacks the original strength of uninjured skin (Singer and Clark, 1999).

Cytokine /Growth Factor	Source/Cell Type	Target Cells and Cellular Effects	Presence in SSc
EGF	Platelets	Keratinocyte motility and proliferation	Elevated
TGF- α	Macrophages, keratinocytes	Keratinocyte motility and proliferation	Elevated
bFGF	Macrophages, endothelial	Angiogenesis and fibroblast proliferation	Elevated
TGF- β 1/ β 2	Platelets, macrophages	Keratinocyte migration, chemotaxis of macrophages and fibroblasts, ECM synthesis and remodelling	Elevated
TGF- β 3	Macrophages	Anti-scarring properties	Reduced
CTGF	Platelets, fibroblasts	Angiogenesis, fibroblast proliferation, ECM synthesis	Elevated
PDGF	Platelets, macrophages, keratinocytes, fibroblasts	Fibroblast proliferation and chemotaxis, macrophage chemotaxis and activation	Elevated
VEGF	Keratinocytes, macrophages	Angiogenesis	Elevated
IL-1 and TNF- α	Neutrophils, Macrophage	Activators of growth factor expression in macrophages, keratinocytes and fibroblasts	Elevated
IGF-1	Fibroblasts, keratinocytes	Reepithelialisation and granulation tissue formation	Unknown

Table 1.4

Cytokines and growth factors in wound healing and SSc.

Adapted from Singer *et al.* and Ihn (Ihn, 2005; Singer and Clark, 1999).

1.2.7 The SSc fibroblast

Seminal studies by LeRoy in the early 1970s demonstrated that skin fibroblasts from patients with SSc cultured *in vitro* were highly active and produced increased amounts of type I collagen compared with fibroblasts from age and sex matched healthy individuals (LeRoy, 1972; LeRoy, 1974). In the intervening years studies with SSc fibroblasts have formed a major basis by which researchers have sought to understand the biochemical and molecular mechanisms that underlie fibrosis in SSc. Subsequent studies have confirmed SSc fibroblasts in culture maintain their 'fibrogenic' phenotype over-expressing many of the constituents of the ECM including GAGs, type III, VI, and VII collagens and fibronectin. The increased biosynthesis of these macromolecules is generally accompanied by an increase in mRNA transcripts predominantly a result of increased gene transcription (Kuroda and Shinkai, 1997; Vuorio et al., 1991; Jimenez et al., 1986; Kahari et al., 1988). In addition, SSc fibroblasts exhibit dysregulated expression of genes key to matrix turnover and processing. SSc dermal fibroblasts (SDF) express elevated levels of TIMP-1 and TIMP-3 and conversely reduced levels of MMP-3 (Mattila et al., 1998; Bou-Gharios et al., 1994). Elevated expression of TIMPs by SDF correlates with increased levels in serum of SSc patients. Increases in levels of TIMP-1 are associated with adverse disease severity (Toubi et al., 2002; Young-Min et al., 2001), and elevated levels of TIMP-2 are associated with an increased risk of cardiac fibrosis (Dziankowska-Bartkowiak et al., 2005; Yazawa et al., 2000). SDF express elevated mRNA levels of lysyl hydroxylase isoform 2 (PLOD2) whose actions are essential for intermolecular collagen crosslinks. Expression of this lysyl hydroxylase is correlated with increased pyridinoline cross-link levels in matrix deposited by SDF (van der Slot et al., 2003).

1.2.7.1 Fibroblast Heterogeneity

The generation of SSc dermal fibroblast lines from biopsies, though relatively easy, has several limitations. The properties of primary cell lines are comparatively variable from different individuals, which may reflect the site of biopsy, stage and heterogeneity of the disease. Furthermore, cell lines have a tendency to revert to a normal phenotype or senesce on prolonged passage in tissue culture (Krieg et al., 1985). This reversion to a 'normal' cell type after several passages probably indicates a loss of phenotype due to successive subcultures or overgrowth by another population of fibroblasts. *In situ* hybridisation of scleroderma skin employing collagen cDNAs demonstrated elevated collagen mRNA transcripts in subpopulations of fibroblasts in contrast to normal skin

(Kahari et al., 1988). Subsequent studies demonstrate the SDF comprise a heterogeneous population of cells (Jelaska and Korn, 2000; Xu et al., 1998b; Rajkumar et al., 2005). SDF are more resistant to Fas induced apoptosis than normal fibroblasts thus a change in ECM expression may arise from the persistence of other cell types such as myofibroblasts (Jelaska and Korn, 2000). The *in vivo* and *in vitro* presence of myofibroblasts in the affected dermal areas of dcSSc patients has been widely reported (Jelaska and Korn, 2000; Kirk et al., 1995; Rajkumar et al., 2005). Myofibroblast differentiation is promoted by several factors elevated in SSc, including ET-1 and TGF- β (Shi-wen et al., 2004; Garrett et al., 2004). Recent *in vitro* studies have shown CTGF to be necessary for TGF- β 1 induced myofibroblast differentiation (Garrett et al., 2004). In addition, increased collagen biosynthesis by myofibroblasts derived from SSc skin has been reported *in vitro* and has led to the hypothesis that the formation and persistence of these cells may represent a key aspect of the disease (Jelaska and Korn, 2000). The recent identification of other mesenchymal cell types derived locally and in circulation that are able to differentiate or perform at least some of the functions of the resident fibroblasts and myofibroblasts cells may represent further sources that contribute to fibrosis (Chesney and Bucala, 2000; Hashimoto et al., 2004).

Locally derived progenitor cells

Pericytes are specialised mesenchymal cells of mesodermal origin, intimately associated with the walls of small blood vessels and modulating endothelial cell function (Shepro and Morel, 1993). Previous studies have shown microvascular pericytes become activated and express platelet-derived growth factor-beta (PDGF- β) receptors in dcSSc skin (Rajkumar et al., 1999). This same report showed activation in autoimmune Raynaud's phenomenon; thus it has been postulated that these cells represent a link between vascular damage and fibrosis.

The acquisition of a mesenchymal phenotype by epithelial cells or epithelial mesenchymal transition (EMT) is recognised as a normal process in cardiac cushion development (Ma et al., 2005). EMT has been widely reported in tumor metastases (Huber et al., 2005) and more recently has been implicated in fibrosis. Although the majority of reports relate to this phenomenon in the development of kidney fibrosis (Zeisberg et al., 2003; Kasai et al., 2005) other studies suggest this may be a feature in the development of fibrosis in other organs (Kasai et al., 2005). The precise contribution of EMT in the pathology of SSc remains to be tested.

Circulatory and Bone Marrow derived cells

Fibrocytes are circulatory fibroblastic cells, with a distinct cell surface phenotype (collagen+/CD13+/CD34+/CD45+) which are recruited to sites of injury (Abe et al., 2001). In addition to collagen type I these cells express inflammatory cytokines and growth factors found within wounds including TGF- β 1 (Chesney et al., 1998). As a result it has been postulated that they may contribute to fibrosis (Bucala et al., 1994; Chesney and Bucala, 2000). Interestingly these same cells have been shown to possess antigen presentation capabilities and may represent a link between inflammation and fibrosis (Chesney et al., 1998; Chesney et al., 1997). More recently Hashimoto *et al.* have shown a population of bone-marrow derived cells contribute to over 80 percent of the collagen expressing cells in bleomycin insulted lungs (Hashimoto et al., 2004). Although *in vitro* these cells are phenotypically similar to fibrocytes, unlike fibrocytes they are insensitive to TGF- β 1 induction of α -SMA (Hashimoto et al., 2004; Abe et al., 2001). As a consequence these cells may represent a population distinct to that of fibrocytes.

1.2.7.2 Gene expression profiling of SSc and SSc fibroblasts

Using explanted fibroblasts from areas of involved and un-involved fibrosis, as well as whole skin biopsies, a range of approaches, including subtractive hybridisation and gene microarrays, have been employed to identify differentially expressed genes in SSc. Increasingly, microarray expression profiling has been used to assess the abundance of thousands of transcripts simultaneously, allowing for an un-biased approach and may reveal novel processes previously unrecognised. The studies thus far have, perhaps unsurprisingly, demonstrated clear differences in gene expression signatures from biopsies and fibroblast cell lines derived from fibrotic involved areas of SSc patients and controls. In addition these studies apparently support the hypothesis that un-involved skin is sub-clinically abnormal (Zhou et al., 2001; Whitfield et al., 2003; Zhou et al., 2005b; Shi-wen et al., 2000). Fibroblasts derived from these regions share many of the differentially expressed genes exhibited by involved skin compared to normal fibroblasts (Claman et al., 1991; Shi-wen et al., 2000; Zhou et al., 2005b).

Whitfield *et al.* investigated gene expression pattern in skin biopsies of four patients with SSc with dcSSc and four normal controls. Genes characteristically expressed in fibroblasts, endothelial cells and B lymphocytes showed differential expression between scleroderma and normal biopsies (Whitfield *et al.*, 2003). Interestingly no gene expression difference was found between explant cultured fibroblasts from scleroderma and normal controls. In contrast Shi-wen *et al.* and others have reported significant differences between in the level of expression of genes between these two clinically defined regions (Shi-wen *et al.*, 2000; Leask *et al.*, 2002a). Studies of early passage nonlesional SDF (n=21) and healthy controls (n=18) by Tan and colleagues found less than 400 of the 8500 genes screened to be differentially expressed (Tan *et al.*, 2005). Analysis of these genes revealed several gene ontology classes that were significantly dysregulated in SSc fibroblasts. These included extracellular matrix structural constituents, cell migration, angiogenesis and complement activation (Tan *et al.*, 2005). Perhaps the strongest study thus far, using these technologies, has come from the gene expression profiling of monozygotic (n=10) and dizygotic (n=5) twins where one twin has SSc (Zhou *et al.*, 2005b). Zhou *et al.* found 40-50% concordance rate for gene expression profiles in dermal fibroblasts from monozygotic twins discordant for SSc. In addition none of the dizygotic twin samples showed concordance for the SSc gene expression profile. Gene clustering analysis identified that monozygotic twins share an ECM gene cluster which is absent in unaffected dizygotic twins. They concluded that healthy fibroblasts from monozygotic twins exhibit a “pre-SSc” stage.

Interestingly even though many of the genes reported to be elevated in SDF are TGF- β responsive genes, TGF- β has not been consistently reported to be elevated in these studies (Zhou *et al.*, 2001; Whitfield *et al.*, 2003; Zhou *et al.*, 2005b; Shi-wen *et al.*, 2000). Zhou *et al.* has proposed that a cellular “memory” of TGF- β is maintained by these cells and thus TGF- β responsive genes such as COL1A2, SPARC, and CTGF are elevated in SDF (Zhou *et al.*, 2005b). The same study found these same genes are upregulated in the unaffected monozygotic twins. Media transfer from dermal fibroblasts from either twin induced an increase in the expression in normal fibroblasts of genes (COL1A2, COL3A1, TIMP3, SPARC and CTGF) typically elevated in SSc fibroblasts. This study supports the idea that a secretable factor expressed by unaffected and affected dermal fibroblasts regulates the expression of these genes. Indeed CTGF

has previously been shown to induce COL1A2 (Shi-wen et al., 2000), and act in an autocrine manner (Riser et al., 2000; Wang et al., 2003).

A recent study by Gardner *et al.* compared mRNA expression of 9 biopsies obtained from the distal forearm of involved skin from SSc patients to that of age and site matched controls. Biopsies exhibited around 1800 gene differences between SSc and normal skin biopsies, with prominent differences in genes associated with the TGF- β , Wnt, ECM proteins and CCN family (Gardner et al., 2006). SSc fibroblasts cultured from the same patients exhibited far fewer gene differences than those observed in biopsies. This may be due to the variety of cells present within the dermis, such as the endothelial cells and pericytes that may be key to driving the differential expression of genes in fibroblasts. Alternatively it may imply that abnormal matrix and matricellular proteins in the SSc environment *in vivo* contribute to the dysregulated expression of genes by the resident fibroblasts. Interestingly although studies by Whitfield *et al.* noted a strong B-cell gene expression signature (Whitfield et al., 2003), this study found no B-cell or immune cell signature (Gardner et al., 2006).

1.2.7.3 Soluble mediators of the SSc fibroblast phenotype

A large body of *in vivo* and *in vitro* work has shown that the production and metabolism of ECM components by normal fibroblasts is modulated by the interplay of soluble factors important in the wound healing process such as PDGF, Insulin-like growth factor (IGFs), endothelin, Tumour necrosis factor- α (TNF- α), CTGF and TGF- β . Many of these same factors have been associated with the pathology of SSc (Table 1.4) and are likely to play critical roles in the initiation and perpetuation of the fibrogenic phenotype of the SSc fibroblast (Leask et al., 2002b; Ihn, 2005).

Platelet derived growth factor (PDGF) is produced by a variety of cell types including fibroblasts, stimulating the production of ECM components, in addition to migration and proliferation of fibroblasts (Bonner, 2004). Levels of PDGF are increased in the bronchoalveolar lavage (BAL) fluids from patients with SSc, and expression of PDGF and its cognate receptors are elevated in skin and mesenchymal cells of SSc patients (Rajkumar et al., 1999; Gay et al., 1989). Over-expression of PDGF in SDF is dependent upon interleukin-1 α (IL-1 α), which is itself constitutively expressed by these cells (Kawaguchi et al., 1999). PDGF may indirectly promote fibrosis through the induction in SDF of the chemokine monocyte chemoattractant protein 1 (MCP-1), a

potent mononuclear cell chemoattractant (Distler et al., 2001). In addition recent studies demonstrate PDGF enhances ERK activation in fibroblasts in conjunction with ROS and thus may directly contribute to the constitutive activation of ERK1/2 and increase in ECM gene expression observed in SDF (Svegliati et al., 2005).

Endothelin-1 (ET-1) has been implicated in the pathogenesis of SSc. In addition to its role as a potent vasoconstrictor, ET-1 enhances proliferation and synthesis of collagen type I and III, whilst repressing the expression of MMP1. ET-1 also promotes myofibroblast differentiation of normal fibroblasts (Xu et al., 1998a; Shi-wen et al., 2004). In SSc, ET-1 and its receptors are elevated on dermal fibroblasts (Shi-wen et al., 2004; Xu et al., 1998b). Bosantan, the ET-1 receptor antagonist, is successfully used in the treatment of PAH in SSc patients. The mode of action of this antagonist is unclear and although primarily believed to inhibit vasoconstriction may inhibit further ECM deposition by mesenchymal cells (Varga, 2003).

Insulin-like growth factors (IGFs) promote the proliferation of normal fibroblasts and the elevated levels of IGFs are, in part, responsible for the increased mitogenic effects of BAL fluid from SSc patients (Harrison et al., 1994). In addition mRNA levels of several IGF-binding proteins (IGFBPs) are consistently elevated in SDF (Shi-wen et al., 2000; Gardner et al., 2006). However the role of IGFs or their binding proteins in the pathobiology of SSc remains unclear.

Tumor necrosis factor-alpha (TNF α) is recognised as a major pro-inflammatory mediator. TNF α inhibits the synthesis of ECM components including collagen type I (Verrecchia et al., 2002) and fibronectin (Kahari et al., 1992) and promotes fibroblast proliferation in a PDGF dependent manner (Battegay et al., 1995). TNF α is a potent inducer of IL-1 β production in several cell types, including fibroblasts. TNF α is also able to suppress the CTGF expression induced by TGF- β in normal and SSc fibroblasts. However, fibroblasts derived from SSc skin tend to be less responsive to this suppressive process (Abraham et al., 2000).

Since TGF- β 's identification in 1981 a growing body of work has demonstrated it to be a major effector of fibroblast biology (Roberts et al., 1981; Verrecchia et al., 2001a; Postlethwaite et al., 1987; Shi-wen et al., 2000; Massague, 1990; Desmouliere et al., 1993). Considered to be the most potent pro-fibrotic cytokines, the TGF- β family is

comprised of three highly conserved mammalian isoforms, TGF- β 1, - β 2 and - β 3 (Sporn and Roberts, 1992; Massague and Wotton, 2000; Massague, 1998). Total TGF- β 1 levels have been reported to be elevated in BAL fluids of SSc patients (Ludwicka et al., 1995) and in serum of dcSSc patients (Snowden et al., 1994). By contrast, a recent study found reduced circulatory levels of active TGF- β 1 in dcSSc patients which correlated negatively with the extent of skin sclerosis (Dziadzio et al., 2005a). Immunohistochemistry and *in situ* hybridisation studies of TGF- β expression have been inconclusive (Sfikakis et al., 1993; Gruschwitz et al., 1990). Work by Kreig's group has shown TGF- β expression to be associated with the inflammatory (leading edge) stage rather than involved fibrotic skin of SSc patients (Querfeld et al., 1999). *In vivo* TGF- β expression in the skin of SSc patients is localized to a number of cell types including fibroblasts, macrophage and endothelial cells (Gruschwitz et al., 1990; Gabrielli et al., 1993).

The role of TGF- β in development and maintenance of the 'fibrogenic' phenotype of SDF in culture is unclear. Kikuchi and colleagues have shown SDF to be mitogenically hypersensitive to TGF- β (Kikuchi et al., 1995), whereas several other groups have reported these cells to be relatively insensitive to further induction of collagen synthesis by TGF- β (Kawakami et al., 1998; Kikuchi et al., 1992; McWhirter et al., 1994). SDF do not express increased levels of TGF- β mRNA or protein compared to normal cells (Ihn et al., 2001b; McWhirter et al., 1994; Gardner et al., 2006). SDF overexpress T β RI and T β RII receptors, which in normal fibroblasts activate the collagen gene promoter (Kawakami et al., 1998; Ihn et al., 2001b). In addition the TGF- β accessory receptors endoglin (Leask et al., 2002a) and betaglycan (personal observation) are elevated on SDF. Results from studies investigating the effects of TGF- β ligand and receptor inhibition have been far from consistent, appearing to affect a sub-set of SSc cell lines exhibiting over-expression of collagen (Ishida et al., 2006; Shi-wen et al., 2006a; Ihn et al., 2001b; Pannu et al., 2004). These studies may support the notion of ligand independent activation of down stream signalling pathways, such as Smads. Indeed several studies of SDF, subsequently performed to those presented within Chapter 4, have found variable differences in components of the Smad signalling pathway (Asano et al., 2004b; Dong et al., 2002; Mori et al., 2003). These studies form the basis of further discussions in Chapter 4. Collectively these studies suggest TGF- β and down stream pathways are likely to play a key role in the initiation of fibrosis, although its precise role in the maintenance of the 'fibrogenic' phenotype of SSc remains unclear.

The absence of TGF- β expression and the cellular 'memory' of TGF- β by SDF have led some researchers to seek downstream modulators of TGF- β fibrotic actions. One such factor known to induce ECM synthesis is CTGF (Shi-wen et al., 2000).

1.2.7.4 Dysregulation of transcription in SSc fibroblasts

The underlying signalling pathways and transcription factors involved in maintaining the 'fibrogenic' features of the SSc fibroblast remain unknown. Strikingly SDF remain active when deprived of serum (Trojanowska et al., 1988; Shi-wen et al., 2000). Trojanowska *et al.* noted SDF display enhanced expression of proto-oncogenes (such as myb, myc, src and ras *et al.*), which are known to participate in cell proliferation and activation (Trojanowska et al., 1988). Several studies have highlighted a variety of components of signalling pathways which exhibit dysregulation in the SDF. Increased phosphorylation of B-Myb, protein kinase C- delta (PKC- δ), phosphatidylinositol 3-kinase (PI3K) have been reported (Cicchillitti et al., 2004; Asano et al., 2004a; Jimenez et al., 2001). In addition, transcription factors NF- κ B/p50, AP-1/JunB (personal observation), CBF and Sp1 demonstrate altered activities in nuclear extracts from SDF (Cicchillitti et al., 2004; Holmes et al., 2003; Ihn and Tamaki, 2000). Pharmacological inhibitors including mithramycin, an inhibitor of Sp1-like GC binding proteins (e.g. Sp1) and LY294002, an inhibitor of PI3K, have confirmed the functional relevance of these pathways in excessive expression of collagen type I in SSc (McGaha et al., 2002; Ihn et al., 2005; Asano et al., 2004a; Jimenez et al., 2001; Ihn and Tamaki, 2000). Unsurprisingly, given the potent effects of TGF- β on mesenchymal cell function and induction of ECM genes, many studies have focused on the regulation of collagen and the role of down stream signalling pathways including down stream mediators of TGF- β action. The role of Smads in maintaining the fibrogenic phenotype of SSc fibroblasts is far from clear and forms the basis of discussions in Chapter 4. Recent advances in profiling simultaneously the activation status of many signalling pathways from comparatively small amount of protein may represent a key tool in the future in defining the relationship of these dysregulated pathways (de Graauw et al., 2006).

The wide variety of signalling pathways dysregulated in SSc fibroblasts and the normal interplay between pathways have by necessity prompted the requirement to define those signalling pathways which play a primary role in the disease phenotype. Several laboratories have therefore focused their attention on the regulatory elements and transcription factors binding these regions, in genes of functional relevance to the

disease (Jimenez et al., 2001; Holmes et al., 2001; Asano et al., 2004a). In transient expression studies the activities of regulatory regions of these promoters have been defined by linking these regions to reporter genes, such as firefly luciferase. Such experiments have provided information about the activities of the type I collagen genes in fibroblasts. Transient transfection studies of $\alpha 2(I)$ procollagen gene (COL1A2) promoter constructs confirmed transcriptional activity was 2-3 fold higher in scleroderma fibroblasts compared with normal cells, and reflected a similar increase in endogenous mRNA levels (Kikuchi et al., 1992; Shi-wen et al., 2000). Transient introduction of this promoter in the presence of pharmacological inhibitors has further confirmed the importance of several signalling components including PKC- δ and p38 in the over-expression of collagen in SDF (Jimenez et al., 2001; Ihn et al., 2005). Using electromobility shift analysis (EMSA) to investigate the functional interaction of transcription factors with regulatory elements in the collagen promoter has identified increased binding activities of factors including, Sp1 and CBF (Holmes et al., 2003; Saitta et al., 2000). Interestingly these factors have been implicated both in the normal basal expression of this gene and in its induction by growth factors and cytokines (Holmes et al., 2003; Greenwel et al., 1997; Lindahl et al., 2002) and thus elevated transcription may represent a general increase in basal transcription rather than the effect of extracellular factors, such as growth factors.

1.3 Animal models of SSc

Animal models have assisted in the understanding of the processes which may contribute to the development of this uncommon disease. Several naturally occurring animal models, both induced and those generated transgenically, are used to study the basic disease processes. Although each model exhibits fibrosis, only some of the specific aspects of the scleroderma pathogenesis, such as vascular damage, inflammation or tissue fibrosis, are represented. The animal models summarised in Table 1.5 represent attractive models to test the potential of disease intervention drugs and therapies.

1.3.1 Naturally occurring models

Two naturally occurring animal models have been described: the tight skin mouse (Tsk1/+ mouse) models and the UCD 200 chicken (Gershwin et al., 1981; van de Water J. et al., 1984; Green et al., 1976).

Tight skin mouse

The tight skin (Tsk1) mouse carries a spontaneous autosomal dominant mutation discovered at the Jackson Laboratory in 1967. Whereas the Tsk1/Tsk1 genotype is embryonically lethal, with embryos dying after 8 days of gestation in utero, the heterozygous (Tsk1/+) mice develop marked thickening of the skin when they are around 2 weeks of age (Green et al., 1976). Although the cutaneous fibrosis is similar to that observed in SSc, Tsk1/+ mice lack the vascular defects present in SSc pathology and develop an enlarged heart. In addition Tsk1/+ mouse lungs display a similar histology to that of human emphysema, rather than the fibrosing alveolitis present in SSc patients (Saito et al., 1999a). The Tsk1 mouse mutation arises as a result of a large intragenic duplication (exons 17-40) of the fibrillin-1 (Fbn1) gene that leads to an abnormally large protein (Kielty et al., 1998). The underlying mechanism by which this duplication alters the matrix metabolism in the Tsk1 mutant mouse is unclear. However Saito *et al.* have shown this duplicated region in the mutant fibrillin, which shares similarities to TGF- β binding protein domains, binds more of the latent form of this fibrogenic growth factor TGF- β (Saito et al., 1999b). The involvement of the immune system in the development of the Tsk1/+ fibrotic phenotype remains unknown. Early studies involving the transplantation of Tsk1/+ bone marrow, T and B lymphocytes into normal mice suggest that fibrosis is transferable (Phelps et al., 1993; Saito et al., 1999a). More recent studies using Tsk1/+ mice on an immuno-deficient background suggest

fibrosis develops independently of mature T and B cells (Dodig et al., 2001). Consequently the immune system may not be required or contribute in the development of fibrosis, at least in this animal model.

UCD 200 line chicken

The University of California Davis (UCD) line 200 White Leghorn Chickens spontaneously develops an inherited scleroderma-like disease with immune vascular and fibrotic characteristics. The UCD line 200 shares many features with human SSc, including skin and visceral fibrosis, vascular occlusion, lymphocyte infiltration in involved organs, elevated rheumatoid factor, antinuclear antibodies and polyarthritis (Gershwin et al., 1981). Immuno-histochemical analysis of UCD 200 chicken skin sections show that the apoptosis of endothelial cells is an early event in pathogenesis preceding the mononuclear perivascular infiltration and collagen deposition (Nguyen et al., 2000). More recent observations suggest endothelial induced apoptosis in this model is mediated by anti-endothelial cell antibodies (Worda et al., 2003). This supports the hypothesis that auto-antibodies to intra-cellular proteins and endothelial cell apoptosis are primary events in the disease process. Apoptotic endothelial cells from fibrotic human skin have been detected in the early inflammatory disease stages of SSc (Sgonc et al., 2000). The UCD 200 appears to be good model for the early vascular aspects of the SSc disease process and supports the theory of vascular damage being an early event in disease progression.

1.3.2 Induced animal models

Murine bleomycin-induced sclerosis

Bleomycin is an antibiotic derived from *Streptomyces verticillus* which is used for the treatment of cancer. Pulmonary fibrosis has been described as an adverse side effect of administration of bleomycin in cancer, and was subsequently used to induce fibrosis in mice. The fibrogenic effects of this drug may be secondary to a free radical injury. The common features of the bleomycin induced fibrosis model with that of SSc are the immune dysregulation, including positive antinuclear antibodies, and upon local instillation the development of dermal and lung fibrosis (Yamamoto et al., 1999b; Nakao et al., 1999; Yamamoto et al., 1999a).

Murine model of fibrosis with graft vs. host disease

Patients that undergo heterologous bone marrow transplantation sometimes develop a chronic graft vs. host disease (GvHD) with skin and visceral fibrosis that resembles scleroderma (Remberger et al., 2003). Transplantation of donor bone marrow into lethally irradiated recipient mice with minor histocompatibility differences results in the development of skin and pulmonary fibrosis 14 to 21 days after transplantation and is reminiscent of the fibrosis which develops in human SSc. In addition the presence of cutaneous immune cells, up-regulation of TGF- β , increased pro(α 1) I collagen mRNA and type I collagen synthesis in the skin have also been demonstrated (Zhang et al., 2002; Zhang et al., 2003). Although the vascular and serologic components of the human scleroderma are not present, the murine GvHD model has proven to be a useful model for SSc.

1.3.3 Transgenic animal models

Mutant fibrillin transgene

Identification of the intragenic duplication of the Fbn-1 gene in the Tsk1/+ mouse led to the development of a transgenic mouse containing this duplicated region. These transgenic animals, like Tsk1/+ mice, develop auto-antibodies including anti-topoisomerase and anti-Fbn1 antibodies as well cutaneous hyperplasia (Saito et al., 2000). However, these transgenics did not develop lung emphysema, suggesting that the Tsk phenotype to be more complex than initially thought.

TGF- β receptor models

Two further transgenic models have targeted the TGF- β receptors. Utilising the Cre LoxP system of controlling gene expression, constitutively active human type I TGF- β receptor (T β RI CA) expression has been targeted to mesenchymal cells using the pro- α 2 (I) collagen enhancer. Histological examination of skin indicates a generalized fibrosis and in addition cultured dermal fibroblasts exhibit elevated gene expression of TGF- β targets (Sonnylal S and de Crombrughe, 2004). Furthermore, using the same mesenchymal lineage-specific expression cassette, Denton *et al.* expressed a kinase-deficient human type II TGF- β receptor (T β RII Δ k) in fibroblasts of transgenic mice to disrupt TGF- β signalling. The resultant transgenics spontaneously developed both skin and lung fibrosis (Denton et al., 2003). The development of fibrosis in other organs and auto-antibodies remains unexplored in both these models. The development of fibrosis in transgenics containing constitutively active T β RI and the apparent counterintuitive

effect of over-expressing a dominant negative T β RII receptor model support a direct role for perturbed TGF- β signalling in fibrosis.

CTGF over-expression

Using the COL1 α 2 enhancer and minimal promoter to target gene expression to mesenchymal cells (Ponticos et al., 2004) a CTGF over-expression transgenic has been generated (Sonnylal, personal communication). Preliminary histological analysis reveals these transgenic animals exhibit dermal thickening and excess matrix deposition in the kidney and lung (Bou-Gharios, personal communication). The precise *in vivo* mechanisms by which CTGF over-expression promotes elevated matrix remain to be clarified. Although explanted fibroblasts from these animals exhibit no significant increase in responsiveness to TGF- β 1, as determined by a Smad-responsive reporter construct (personal observation), the molecular mechanisms remain to be determined.

Parameters	Human SSc	Tsk/+ mouse	Murine Bleo induced	Murine SSc GvHD	TGF- β receptor tg	UCD 200	CTGF tg
Skin fibrosis	+	+	+	+	+	+	+
Visceral fibrosis	+	+	+	+	?	+	?
Pulmonary	+	-	+	+	?	+	+
Renal	+	-	-	-	?	+	+
Cardiac	+	+	-	?	?	+	?
Inflammation	+	-	+	+	?	+	?
Vascular injury	+	-	+	-	?	+	?
Auto-antibodies	+	+	+	-	?	+	?

Table 1.5

Animal models of SSc.

SSc GvHD= Sclerodermatous graft vs. host disease UCD 200= University of California at Davis 200 chicken. Bleo=Bleomycin induced; tg= transgenic. Modified from Zhang and Gilliam (Zhang and Gilliam, 2002)

1.4 Connective Tissue Growth Factor

1.4.1 The CCN Family

The characterisation of two structurally related gene products, namely cysteine-rich 61 (cyr61) and connective tissue growth factor (CTGF), in the mid-1980s led to the identification of a family of conserved modular proteins (Almendral et al., 1988; Lau and Nathans, 1985; Brunner et al., 1991). The CCN family, named for the founding members CYR61, CTGF and Nov, comprises six distinct genes in humans (Cyr61, CTGF, Nov, WISP1-3). CCN orthologs have been identified in a range of vertebrate species including *xenopus*, chicken and mouse. In 2003 the nomenclature of the CCN family was unified (Figure 1.2) to reflect the order of each family member's discovery (Brigstock et al., 2003).

Biologically the CCN family of proteins mediates diverse cellular effects, including angiogenesis, chondrogenesis and wound healing (Nakanishi et al., 2001; Perbal et al., 2003; Mo et al., 2002; Ivkovic et al., 2003). In addition CCN family members have been implicated in several human pathologies including tumourgenesis and fibrosis. For example, Nov was originally isolated from nephroblastomas (Joliot et al., 1992), and mutations in WISP3/CCN6 are associated with progressive pseudorheumatoid dysplasia (Hurvitz et al., 1999).

Encoded by up to 5 exons, the cysteine-rich CCN proteins share between 40-60% nucleotide and 30-50% amino acid homology (Lau and Lam, 1999). In addition to an amino-terminal signal peptide sequence encoded by exon 1, the CCN proteins contain up to four structural modules (Figure 1.2). Although the crystal structure of CTGF is unknown, each module exhibits significant sequence and structural homology to conserved regions found in a number of extracellular proteins (Moussad and Brigstock, 2000); an N-terminal region similar to the insulin-like growth factor binding protein is encoded by exon 2 [**module 1**]; a von Willebrand factor type C repeat is encoded by exon 3 [**module 2**]; a thrombospondin type I motif, and the cysteine knot within the C-terminal module similar to that found in TGF- β and PDGF is encoded by exon 4 [**module 3**]; Exon5, absent in CCN5, shares homology with several extracellular proteins and is believed to mediate protein-protein interactions or dimerisation [**module 4**]. These protein modules act both independently and interdependently in promoting the functional effects of the CCN proteins (Perbal et al., 2003). Separating modules 2 and 3

is a variable 'hinge' region which is susceptible to cleavage by proteases (Hashimoto et al., 2002). Post-translational processing or alternative splicing lends further complexity to the family members. Variant proteins lacking one or more module have been identified in both normal and pathological situations. For example, amino-terminal-truncated forms of CCN3, identified in nephroblastomas, are sufficient to induce the transformation of chicken embryo fibroblasts (Joliot et al., 1992). The precise sites of expression and the biological implication of CCN truncations and cleavage products remain unclear. The independent and interdependent actions of these protein modules suggest that the generation of truncated forms of CCN family members is likely to play a significant biological role. Indeed a recent report has shown that the C-terminal domain (Modules III and IV) of CTGF appear to regulate fibroblast proliferation whereas the N-terminal domain (Modules I and II) mediates myofibroblast differentiation and collagen synthesis (Grotendorst and Duncan, 2005), the latter being elevated in circulation in systemic sclerosis patients (Dziadzio et al., 2005b).

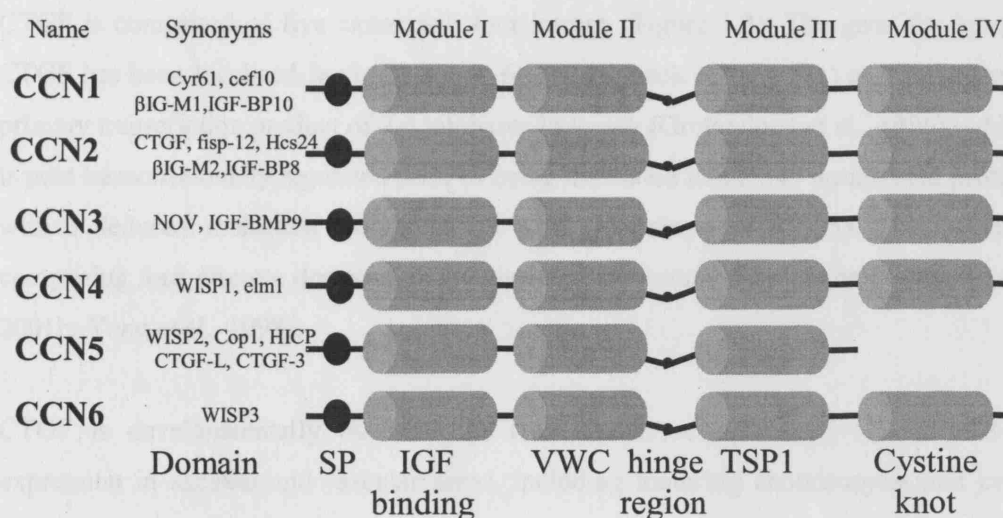


Figure 1.2

Schematic representation of the CCN protein structure.

The nomenclature of the CCN family was unified in 2003 to reflect the order of each family member's discovery (CCN1-6). The multimodular structure is comprised of: SP=secretory peptide; insulin-like growth-factor-binding domain; VWC= von Willebrand domain; TSP1= thrombospondin-like domain; Cysteine knot dimerisation domain. At the junction between module II and III is a region referred to as the hinge region. Modified from Leask *et al.* and Perbal *et al.* (Leask and Abraham, 2003; Perbal, 2004).

1.4.2 Identification of CTGF

The murine ortholog of CTGF, fibroblast-inducible secreted protein-12 (fisp-12), was first identified in serum stimulated fibroblasts in 1988 (Almendral et al., 1988). Differential screening of TGF- β 1 induced genes independently identified a homologous transcript in AKR-2B cells (Brunner et al., 1991). In the same year, the term 'connective tissue growth factor' was first used by Bradham *et.al* to describe a novel growth factor secreted by cultured human umbilical vein endothelial cells (Bradham et al., 1991). CTGF represents a prototypical member of the CCN family, promoting both direct cellular effects and adaptive cellular functions i.e. altering the cellular response to other extracellular stimuli. CTGF is an atypical growth factor sharing several characteristics of the matricellular proteins, thrombospondin-1 (TSP1), secreted protein, acidic, rich in cysteine (SPARC; osteonectin), and osteopontin (OPN) (Bornstein and Sage, 2002; Brigstock et al., 2003).

1.4.3 CTGF gene expression

CTGF is comprised of five exons and four introns (Figure 1.3). The gene for human CTGF has been localized to chromosome 6q23.1 (Ryseck et al., 1991) and generates a primary transcription product of 2.4 kilobases in length (Grotendorst et al., 1996), which is post transcriptionally regulated prior to being translated into a 349 amino acid protein with a deduced molecular weight of 38 KDa (Kubota et al., 2005). This protein, comprising four discrete domains, is glycosylated and secreted by the cell (Chen et al., 2001b; Yang et al., 1998).

CTGF is developmentally essential; *in vivo* studies reveal strong CTGF mRNA expression in skeletal and vascular areas, including maturing chondrocytes and cells within the heart (Ivkovic et al., 2003). In the adult, CTGF gene expression is more restricted and present at high levels in the mesenchyme of the cardiovascular system and the ovarian follicles or testicular tubes (Friedrichsen et al., 2005). CTGF expression is rapidly induced in the mesenchyme as a result of trauma, for example during adult tissue repair (Igarashi et al., 1993). CTGF protein has also been detected in biological fluids including bronchoalveolar lavage, interstitial and tear fluids, serum, and urine (Shi-wen et al., 2000; Dziadzio et al., 2005b; van Setten et al., 2003; Sato et al., 2000; Riser et al., 2003). *In vitro*, CTGF is transcriptionally expressed by a variety of cell types including dermal fibroblasts, smooth muscle cells, mesangial cells, renal

podocytes, chondrocytes, pancreatic ductile cells, pericytes, endothelial cells and T-lymphocytes (Shi-wen et al., 2000; Grotendorst et al., 1996; Nakanishi et al., 2000; Crean et al., 2004; Roestenberg et al., 2005; Yosimichi et al., 2001; di Mola et al., 2002; Suzuma et al., 2000; Bradham et al., 1991; Workalemahu et al., 2003).

1.4.4 CTGF gene regulation

The expression of CTGF is tightly regulated primarily at the level of gene transcription (Shi-wen et al., 2000; Grotendorst et al., 1996). TGF- β induces CTGF mRNA in fibroblasts within 30 minutes of treatment in a manner that does not require *de novo* protein synthesis. CTGF homologues were originally isolated from fibroblasts stimulated with serum and TGF- β . Subsequent studies have demonstrated several factors which transcriptionally alter CTGF gene expression including VEGF, EGF, ET-1, PDGF, TNF- α , and thrombin (Xu et al., 2004; Suzuma et al., 2000; Igarashi et al., 1993; Abraham et al., 2000; Chambers et al., 2000). In addition CTGF gene expression changes in response to environmental factors including hypoxia, mechanical stress and infection (Higgins et al., 2004; Schild and Trueb, 2002; Unnikrishnan and Burleigh, 2004). Other important regulators of CTGF include reactive oxygen species (ROS) and glucocorticoid (Park et al., 2001; Dammeier et al., 1998a).

Consistent with several extracellular factors which affect CTGF expression, many signalling pathways regulate CTGF gene expression, including protein kinase C (PKC), NF- κ B and members of the MAPK pathway, including ERK and JNK (Brigstock, 1999; Chen et al., 2002; Leask et al., 2003). The mechanisms by which these intracellular signalling pathways regulate CTGF expression are not fully understood, however, CTGF promoter reporter studies have been instrumental in defining several regulatory elements and transcription factors involved (Abraham et al., 2000; Holmes et al., 2001; Holmes et al., 2003; Higgins et al., 2004; Xu et al., 2004; Van Beek et al., 2006). Analysis of the proximal CTGF promoter region (< 1 kilobase 5' of the TATA box) has identified ET-1 and TGF- β response elements (Grotendorst et al., 1996; Xu et al., 2004). The precise transcription factors regulating CTGF expression by ET-1 remain unclear, whereas the mechanism by which TGF- β induces CTGF transcription forms the basis of studies presented in Chapter 3 (Holmes et al., 2001).

The tumour suppressor gene, *WT1*, represses CTGF promoter activity through novel recognition elements (Stanhope-Baker and Williams, 2000). A recent study has shown in chondrocytes the transcription factor c-Maf, whose absence leads to abnormal terminal differentiation of hypertrophic chondrocytes, transcriptionally induces CTGF activation (Omoteyama et al., 2006). In addition to proximal elements, regulatory elements in the distal portion (>1 kilobase from the TATTA box) promoter region have recently been characterised, including a hypoxia response element to which the transcription factor HIF-1 α binds (Higgins et al., 2004), a stretch response element (Chaqour et al., 2006), thrombin response element in addition to several putative transcription factor binding motifs, such as AP-1. The precise mechanism by which CTGF expression is regulated by other factors known to induce its expression still remains unclear. For example, knock out studies suggest CTGF expression is essential in bone development and BMPs are likely to play a key role in the regulation of CTGF (Ivkovic et al., 2003). In silico analysis of the CTGF promoter reveals several BMP/Smad consensus elements, however the functionality of these elements and the effects of BMPs remain unclear. In addition promoter studies performed by Abraham and colleagues have highlighted the importance of crosstalk between signalling pathways activated by extracellular stimuli in the regulation of CTGF (Abraham et al., 2000; Chen et al., 2002; Leask et al., 2003). For example, TGF- β activation of the CTGF promoter is antagonised by TNF- α , in an NF- κ B dependent manner (Abraham et al., 2000). In addition CTGF expression is regulated in a cell specific manner by retinoids (Shimo et al., 2005; Cash et al., 1998) and TGF- β (Phanish et al., 2005; Leask et al., 2003). Given the normally low level of expression of CTGF in fibroblasts, elements within the promoter which regulate the basal expression may be of specific importance to the pathological over-expression of CTGF. One such element, the Sp1-like binding site (Figure 1.3) forms the basis of studies presented in Chapters 5 and 6.

CTGF transcription is further regulated through elements within the 3' untranslated region (UTR) (Figure 1.3). This cis-acting element of structure-anchored repression (CAESER) destabilises CTGF mRNA (Kubota et al., 2005). The effects of this region may account for the apparent conflicting observation that increases in CTGF mRNA by growth factors such as PDGF and EGF do not lead to a comparable increase in CTGF protein (Igarashi et al., 1993). Further studies will be required to clarify the precise mechanisms underlying this apparent dichotomy. Collectively these studies suggest transcriptional control via multiple elements within the CTGF promoter and 3' UTR are

likely to account for the complex and tightly regulated expression of this protein during development and in the adult.

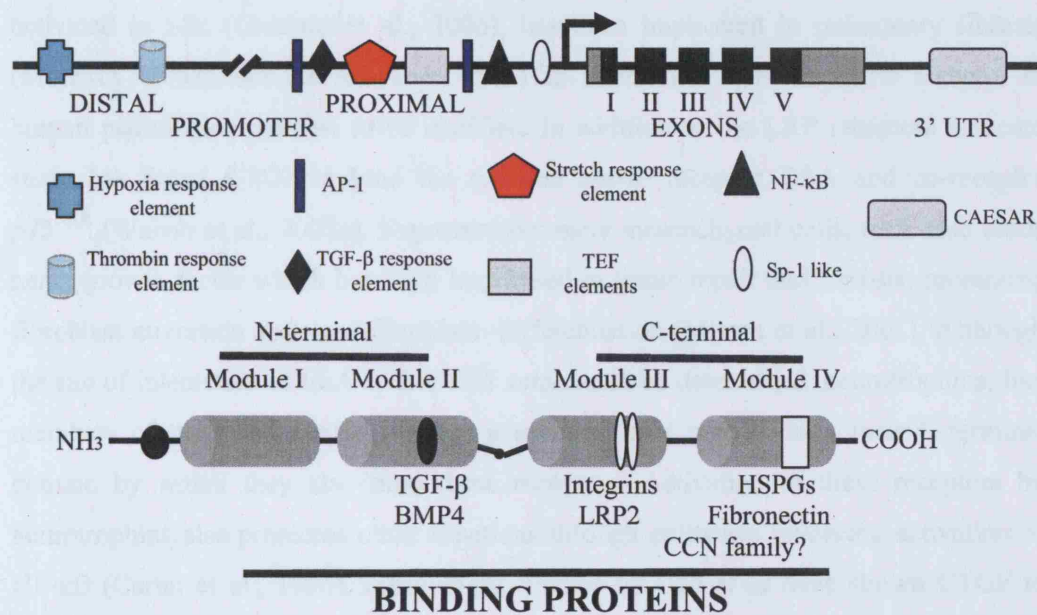


Figure 1.3

CTGF gene and promoter structure.

A schematic of the genomic structure of CTGF indicating known and putative regulatory elements within the non-coding region of CTGF (upper panel). CTGF modular protein arrangement indicating regions of interaction with cellular and extracellular proteins (lower panel). Modified from Leask *et al.* (Leask *et al.*, 2002b)

1.4.5 CTGF receptors and downstream signalling pathways

CTGF interacts with a variety of cell surface molecules; however no unique *bona fide* cellular receptor has been identified (Table 1.6). Like other CCN family members CTGF binds to a variety of integrins primarily through elements within module 3 of the protein including $\alpha 5\beta 1$ and $\alpha 4\beta 1$ (Gao and Brigstock, 2005b; Heng et al., 2006; Hoshijima et al., 2006). CTGF also binds the low density lipoprotein-like receptor 1 via elements contained within this module (Segarini et al., 2001; Yang et al., 2004). In the developing *Xenopus* CTGF represses Wnt signalling through C-terminal interactions with the Wnt co-receptor, LRP6 (Mercurio et al., 2004). Interestingly Wnt signalling, activated in SSc (Gardner et al., 2006), has been implicated in pulmonary fibrosis (Morrissey, 2003), but the relevance of CTGF interaction with the LRP6 receptor in human pathologies remains to be clarified. In addition to the LRP receptors a recent study has found CTGF to bind the tyrosine kinase receptor TrkA and co-receptor p75^{NTR} (Wahab et al., 2005a). Expressed on many mesenchymal cells, trkA also binds nerve growth factor which has been implicated in tissue repair and fibrosis, promoting fibroblast migration and myofibroblast differentiation (Micera et al., 2001). Although the site of interaction of trkA with CTGF remains to be determined, neurotrophins, like members of the CCN family, contain a cysteine knot motif within their C-terminal domain by which they also bind these receptors. Activation of these receptors by neurotrophins also promotes other functions through pathways involving activation of NF- κ B (Carter et al., 1996). Intriguingly, studies by Gao *et al* have shown CTGF to activate the NF- κ B signalling pathway through as yet undefined cellular receptors (Gao and Brigstock, 2005a). Heparin sulphate containing proteoglycans, including syndecan-4 and perlecan, bind the CTGF protein through a heparin binding domain contained within module 4 (Nishida et al., 2003; Chen et al., 2004). Recent studies have shown that CTGF also forms a complex with matrix molecules, such as fibronectin, and their cellular receptors and may function as a adaptor protein to promote the interaction of extracellular proteins to their cellular receptors (Chen et al., 2004). Consistent with this CTGF also binds members of the TGF- β superfamily, promoting TGF- β and antagonising BMP4 binding to their concordant receptors through the cysteine-rich domain in second module of CTGF (Abreu et al., 2002).

The activation of downstream signalling pathways by CTGF may be divided into adaptive and direct effects. CTGF binding to growth factors alters the downstream effects presumably through changes in the kinetics of binding to their receptors or through adapting the cellular signalling. For example, binding of CTGF to TGF- β enhances the downstream activation of Smad2, whereas the absence of CTGF alters the kinetics of TGF- β activation of Akt (Abdel et al., 2005; Shi-wen et al., 2006b). To date, no CTGF specific signalling pathway has been identified; rather reports suggest CTGF directly activates several non-specific pathways, including members of the MAP kinase pathways ERK1/2, JNK and p38 (Yang et al., 2004; Yosimichi et al., 2001; Yosimichi et al., 2006). Other reports indicate CTGF activates NF- κ B, Akt, ERK5, protein kinase C alpha (PKC α) and delta (PKC δ) through a variety of cellular receptors (Gao and Brigstock, 2005a; Wahab et al., 2005a) in addition to promoting changes in intracellular calcium (Li et al., 2002). The activation of multiple signalling pathways may reflect the lack of a specific cell surface receptor and as such account for the reported differences in response to CTGF (Gao and Brigstock, 2005a; Wahab et al., 2005a). Interesting studies by Wahab *et al.* suggest CTGF may also function as an intracellular signalling molecule (Wahab et al., 2001a), adding further complexity to its mechanism of action. Binding of CTGF to an undefined cellular receptor(s) was shown to lead to its internalisation in endosomes and accumulation of CTGF in a juxtanuclear organelle from which the growth factor was found to translocate into the cytosol. Within the cytosol CTGF was found to be phosphorylated by protein kinase C and translocated into the nucleus (Wahab et al., 2001a). The translocation of CTGF induced an increase in transcription within mesangial cells which was attributed to elevated levels of ribosomal mRNA and not of previously reported CTGF modulated genes, such as collagen type I and fibronectin (Wahab et al., 2001a). Although not novel, since a number of other cytokines and growth factors have also been reported to be transported to the nucleus, including the CCN family member NOV (Perbal, 1999), the precise biological relevance of CTGF nuclear translocation remains unknown.

Cellular Receptor	Cell Type	Function	Reference
Integrin			
$\alpha 6 \beta 1$	Fibroblast	Adhesion	(Chen et al., 2001a)
$\alpha v \beta 3$	Endothelial	Adhesion/proliferation/ cell survival	(Babic et al., 1999)
$\alpha IIb \beta 3$	Platelets	Adhesion	(Jedsadayanmata et al., 1999)
$\alpha 5 \beta 1$	Stellate	Adhesion/ migration	(Gao and Brigstock, 2005c)
$\alpha 5 \beta 1$	Mesangial	Matrix deposition	(Weston et al., 2003)
$\alpha M \beta 2$	Monocytes	Adhesion	(Schober et al., 2002)
LRP1	Fibroblasts, Osteoblastic	Adhesion/myofibroblast differentiation	(Segarini et al., 2001; Yang et al., 2004)
LRP6	*	Blocks WNT signalling	(Mercurio et al., 2004)
Perlecan	Chondrocytes	Proliferation	(Nishida et al., 2003)
TrkA	Mesangial	Unknown	(Wahab et al., 2005a)
Syndecan-4	Fibroblasts	Adhesion	(Chen et al., 2004)

Table 1.6

CTGF binds multiple cellular receptors.

CTGF interacts with several cell surface associated proteins through different regions of the modular protein to elicit down stream effects in a variety of cell type. *cell type unknown-inhibits WNT binding to the LRP6 receptor in developing *Xenopus*.

1.4.6 Biological function of CTGF

Since the original identification of CTGF over 15 years ago, the biological function of this protein still remains the focus of intense investigation and debate. The main biological effects ascribed to CTGF are described in Table 1.7. Possibly due to the high cysteine content of CCN proteins, it has proved comparatively difficult to produce pure recombinant CTGF (rCTGF) protein preparations, which has hindered the investigations of the precise mode of action of CTGF (Lau and Lam, 1999; Frazier et al., 1996). *In vivo* expression of CTGF suggests a likely role in chondrogenesis, extracellular matrix remodelling, angiogenesis and wound healing. Consistent with this CTGF deficient mice die soon after birth, and display severe skeletal defects due to grossly impaired ossification (Ivkovic et al., 2003). In these mice the lack of CTGF causes impaired chondrocyte proliferation and reduced expression of the ECM protein, aggrecan, within

the hypertrophic zone (Ivkovic et al., 2003). The hypertrophic zones of CTGF deficient mice are expanded and endochondral ossification is impaired due to decreased expression of VEGF and aberrant vascularization in the hypertrophic zones (Ivkovic et al., 2003). Furthermore skeletal development of transgenic mice which over-express CTGF under the control of mouse type XI collagen promoter is stunted. Phenotypically these mice exhibit dwarfism within a few months of birth possibly as a result of premature endochondral ossification (Nakanishi et al., 2001). In addition, the associated pathological over-expression of CTGF in tumours and fibrotic disorders supports the role of CTGF in these biological functions (Table 1.9). Functionally the cellular mechanisms by which CTGF mediates these effects may be divided into adaptive and direct (Figure 1.4). For example, *in vitro* studies have demonstrated CTGF promotes ECM gene expression (Shi-wen et al., 2000) whereas other studies have highlighted the requirement of CTGF in TGF- β induction of these same effects (Wang et al., 2004).

Adaptive effects of CTGF

The ability of different modules of this multi-domained protein to bind growth factors, ECM proteins and membrane associated proteins has led to the notion that CTGF may function to integrate multiple external stimuli and facilitate cellular responses to these (Figure 1.4). For example CTGF acts as a bridge to promote the interaction of fibronectin and cell surface integrin receptors (Chen et al., 2004). The functional characteristics of CTGF are similar to those associated with matricellular proteins such as SPARC, TSP1 and OPN and this has led to CTGF being proposed as a matricellular protein (Bornstein and Sage, 2002; Leask et al., 2002b).

Perhaps the best studied adaptive action of CTGF is in the regulation of TGF- β function. CTGF is strongly transcriptionally and translationally induced by TGF- β , and has been widely proposed as a downstream mediator of its actions on mesenchymal cells (Shi-wen et al., 2000; Daniels et al., 2003; Wang et al., 2004; Shi-wen et al., 2006b). Recent studies by Shi-Wen *et al.* using embryonic fibroblasts from CTGF null mice found a third of all transcripts induced by TGF- β to be CTGF dependent (Shi-wen et al., 2006b), whereas only a small proportion of basally expressed genes in fibroblasts lacking CTGF appeared to be affected (Leask *et al.* personal communication). The requirement of CTGF for a proportion of TGF- β cellular activities is consistent with CTGF interference siRNA and antisense studies (Daniels et al., 2003; Wang et al., 2004). Considerable efforts have been expended towards understanding the mechanism

by which CTGF affects TGF- β function. The absence of CTGF alters the kinetics of TGF- β 's activation of signalling pathways, such as Akt (Shi-wen et al., 2006b). In addition CTGF, through direct protein-protein interactions, enhances TGF- β 1 binding to its receptors and leads to the enhanced activation of Smad2 in fibroblasts (Abreu et al., 2002). In mesangial cells, CTGF has been shown to promote the sustained induction of a member of the Sp1-like/kruppel family of transcription factors, TIEG-1, an antagonist of the inhibitory Smad, Smad 7 (Wahab et al., 2005b). Thus CTGF blocks the negative feedback loop provided by Smad 7, and leads to the enhancement of activation of Smad2 and Smad3. Interestingly TIEG-1 levels are unaltered in CTGF null fibroblasts and thus CTGF/TIEG-1 repression of SMAD7 may be cell type specific (Shi-wen et al., 2006b). Further CTGF has been reported to induce TSP-1, a matricellular protein which plays a key role in the activation of latent TGF- β (Wang et al., 2001).

CTGF interacts with other proteins, including fibronectin, BMP4 and VEGF(165) (Table 1.8) both promoting and inhibiting their function (Abreu et al., 2002; Chen et al., 2004; Hashimoto et al., 2002). CTGF acts synergistically with other growth factors including EGF, and hormones such as insulin however the manner by which CTGF promotes these effects remains unclear (Gore-Hyer et al., 2003; Frazier et al., 1996). It is also noteworthy that CTGF both forms homomeric complexes, and binds to the CCN family member, NOV (Wahab et al., 2001a). Although the mechanisms are unknown, it is likely that these interactions are mediated via elements within module 4. The ability of CTGF to interact with several other proteins, whose bio-availability may differ depending on organ or biological situations, such as injury, could result in different biological effects and a multifunctional response to CTGF. The mechanism(s) by which CTGF facilitates these cellular processes remains as yet unclear.

Direct effects of CTGF

CTGF is a factor of major importance in reparative processes, in addition to being essential during development. Strong *in vitro* evidence supports a role for CTGF in fibroblast and endothelial cell adhesion through cell surface receptors, including integrins and proteoglycans (HSPGs) (Chen et al., 2004; Babic et al., 1999), and in other cell types, including mononuclear cells and platelets (Schober et al., 2002; Jedsadayanmata et al., 1999). Other reported cellular effects of CTGF in endothelial and mesenchymal cells, summarised in table 1.7, include proliferation, migration, and

apoptosis (Hishikawa et al., 2000; Lau and Lam, 1999; Babic et al., 1999; Grotendorst and Duncan, 2005). In addition in mesenchymal cells CTGF promotes ECM expression, including fibronectin and collagen type I (Weston et al., 2003; Frazier et al., 1996). Of note is a study by Shi-wen *et al.* which identified a CTGF response element in the human COL1A2 promoter between -376 and +17 of the TATAA box (Shi-wen et al., 2000). Interestingly the reporter activity of this promoter region, which also contains a TGF- β responsive element and functional Sp1 site (Zhang et al., 2000), is significantly elevated in SSc fibroblasts, and is not further stimulated by exogenous addition of TGF- β 1 (Kikuchi et al., 1992). Consistent with the role of CTGF in repair, *in vitro* rCTGF transcriptionally induces collagen type I and III, and TIMPs, and the decline of mRNA transcripts of MMPs, in porcine skin fibroblasts (Wang et al., 2003). Induction of these genes was promoted by a C-terminal truncated rCTGF protein whereas DNA synthesis required the full length rCTGF protein (Wang et al., 2003). However, contradictory studies have found in normal rat kidney fibroblasts the C-terminal of CTGF mediates proliferation and the N-terminal to promote collagen synthesis and differentiation of myofibroblasts, a key cell type in wound repair (Garrett et al., 2004; Grotendorst and Duncan, 2005).

In summary, CTGF shares some characteristics of matricellular proteins. Many of the direct functional effects ascribed to CTGF may in fact arise from adapting the cellular responses to endogenously expressed cellular growth factors and cytokines, such as TGF- β . *In vivo* studies involving targeted deletion, inhibition or over-expression support the notion that CTGF plays a key role in development, wound healing, and angiogenesis. The mechanism by which CTGF mediates these processes, be it direct or adaptive, remains unclear. CTGF interacts with other proteins in both a synergistic and inhibitory manner (Table 1.8). The *in vitro* studies performed to investigate CTGF function cannot preclude it acting to enhance cellular response to endogenously expressed growth factors and cytokines. The precise mechanism(s) by which CTGF facilitates these cellular processes remain as yet unclear.

Cell Type	Bioactivity	Reference
Fibroblast, chondrocytes, osteoblasts, endothelial, smooth muscle, mesangial	Chemotaxis, Proliferation	(Crean et al., 2004; Fan et al., 2000; Shimo et al., 1999; Nakanishi et al., 2000; Bradham et al., 1991)
Endothelial, mesangial, smooth muscle, epithelial (MCF-7 cancer cell line)	Apoptosis	(Babic et al., 1999; Brigstock, 2003; Hishikawa et al., 1999; Hishikawa et al., 2000)
Fibroblasts and mesangial	ECM production	(Crean et al., 2002; Grotendorst and Duncan, 2005)
Fibroblasts (NRK cells), mesangial	Cell cycle	(Abdel-Wahab et al., 2002; Kothapalli and Grotendorst, 2000)
Chondrocytes, osteoblasts, fibroblasts	Differentiation	(Nakanishi et al., 2000; Grotendorst and Duncan, 2005)
Fibroblasts and mesangial	Cytoskeletal rearrangement	(Crean et al., 2004; Chen et al., 2004)

Table 1.7

The major biological properties of CTGF.

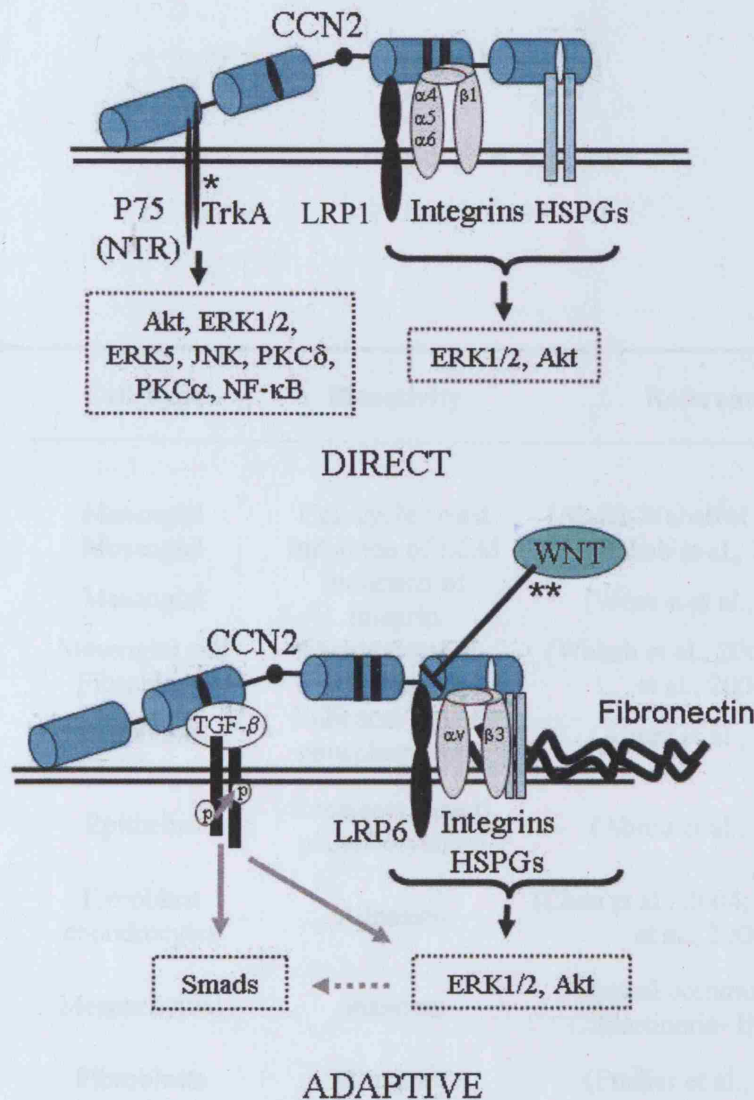


Figure 1.4

The putative mechanism by which CTGF induces transcriptional changes.

(1) **DIRECT:** CTGF binds several cellular receptors activating generic signalling pathways, including ERK and Akt. (2) **ADAPTIVE:** CTGF binds extracellular proteins, including TGF-β and fibronectin, which promote/prolong the activation of non-CTGF induced signalling pathways through alteration of the kinetics of ligand/receptor interactions (Chen et al., 2004; Abreu et al., 2002). In addition CTGF may act to inhibit repressors of non-CTGF induced signalling pathways to further prolong signalling, for example induction of TIEG a repressor of SMAD7 (Wahab et al., 2005b). *The precise mechanism of interaction of CTGF with TrkA and p75 remain unknown (Wahab et al., 2005a). **CTGF binds the WNT receptor LRP6 and inhibits WNT binding and down stream signalling pathways (Mercurio et al., 2004).

Protein	Cell Type	Bioactivity	Reference
<i>TGF-β</i>	Mesangial	Cell cycle arrest	(Abdel-Wahab et al., 2002)
	Mesangial	Induction of ECM	(Wahab et al., 2001b)
	Mesangial	Induction of integrin	(Weston et al., 2003)
	Mesangial and Fibroblasts	Cytoskeletal re-arrangements	(Wahab et al., 2005b; Chen et al., 2004)
	Fibroblasts	Enhances Smad2 phosphorylation	(Abreu et al., 2002)
<i>BMP-4</i>	Epithelial	Represses Smad1 phosphorylation	(Abreu et al., 2002)
<i>Fibronectin</i>	Fibroblast chondrocytes	Adhesion	(Chen et al., 2004; Hoshijima et al., 2006)
<i>NOV</i> (CCN3)	Mesenchymal	unknown	Personal communication C.Martinerie- INSERN
<i>EGF*</i>	Fibroblasts	Mitogenic	(Frazier et al., 1996)
<i>bFGF*</i>	Endothelial	Enhances mitogenic activity	(Kireeva et al., 1997)
<i>VEGF(165)</i>	Endothelial	Angiogenesis	(Inoki et al., 2002)
<i>IGF**</i>		Unknown	(Kim et al., 1997)

Table 1.8

CTGF interacts with extracellular proteins affecting their down stream actions.

CTGF has been reported to interact with several extracellular proteins and modulate their biological activities. *Direct interaction with CTGF remains to be clarified. **Physical interaction with these proteins has been shown though no function has been ascribed.

1.4.7 CTGF and wound healing

Key to the initiation of wound healing and tissue regeneration is coagulation. CTGF is the most abundant growth factor contained within human platelets (Kubota et al., 2004). Recent studies have found that in addition to TGF- β , CTGF is released during coagulation that initiates the wound healing process (Kubota et al., 2004; Liu et al., 2003). Defining the direct *in vivo* role of CTGF in regenerative processes remains a key question. The use of CTGF neutralizing antibodies, or more attractively the generation of conditional knockouts of CTGF in mesenchymal cells should better define the role of CTGF. The subsequent coordinated expression of TGF- β and CTGF has been suggested to represent a growth factor cascade post coagulation, in which TGF- β initiates regeneration and repair and the consequential induction of CTGF is required for the later stages of the repair process (Igarashi et al., 1993). A number of studies have highlighted the likely importance of CTGF in wound healing (Duncan et al., 1999; Stratton et al., 2002; Igarashi et al., 1993). In uninjured skin TGF- β 1 and CTGF transcripts are not detectable by northern blot, however upon injury both are rapidly induced (Igarashi et al., 1993). Wound healing studies by Grotendorst *et al.* have shown TGF- β 1 transcripts peak three days after wounding, whereas CTGF transcripts peak nine days after injury and are predominantly expressed by the connective tissue and vascular endothelial cells present within the granulation tissue (Igarashi et al., 1993). Both these transcripts returned to basal levels on the termination of the reparative process. *In vivo* dermal administration of rCTGF alone promotes the formation of limited granulation tissue, whereas TGF- β alone promoted a transient granulation tissue for 7 days to form. However injecting both growth factors promoted the long-term formation of fibrotic tissue which persisted for 14 days (Mori et al., 1999). This observation suggests the temporal expression of CTGF and TGF- β , in a coordinated manner, is required for faithful wound healing and inappropriate expression of CTGF can result in dysregulated wound healing and fibrosis.

1.4.8 CTGF in human pathologies and disease models

A growing body of clinical evidence supports the role of CTGF in human diseases, particularly in tumorigenic and fibrogenic pathologies (Table 1.9). The role for CTGF in cancer is far less clear and may represent cell type differences in response. CTGF has been shown to affect cell-cycle progression, by up-regulating cyclin A and reducing p27^{kip1} (Kothapalli and Grotendorst, 2000). CTGF has been found to be highly expressed in proliferating endothelial cells and glioma cells (Pan et al., 2002). CTGF mRNA is absent in the breast cancer epithelial cell line, MCF-7. Over-expression of CTGF induces apoptosis in these cells and is consistent with the reduction in cell growth and tumorigenesis observed in over-expressing human oral squamous cell carcinoma-derived cell lines (Hishikawa et al., 1999; Moritani et al., 2003). In prostate cancers, CTGF expression is strongly associated with the surrounding stroma, and has been proposed to act positively in conditioning the local microenvironment of the tumour (Yang et al., 2005). CTGF expression is repressed by the tumour suppressor, WT1, mutations in which are linked to paediatric nephroblastomas (Stanhope-Baker and Williams, 2000).

Perhaps the most persuasive evidence of a pathological role for CTGF arises from investigations into human fibrotic diseases, including keloids, Dupuytren's contracture, and SSc (Table 1.9). To date CTGF has been found to be universally over-expressed in all fibrogenic disorders investigated i.e. there is no fibrosis without an increase in CTGF levels (Table 1.9). For example, in idiopathic pulmonary fibrosis CTGF is transcriptionally and translationally elevated. Expression is confined to proliferating type II alveolar cells and myofibroblasts, which are believed to play a critical role in pulmonary fibrosis (Pan et al., 2001). In atherosclerotic lesions, CTGF is highly expressed by the intimal smooth muscle cells (Oemar et al., 1997). Furthermore CTGF is over-expressed in several established animal models of connective tissue disease, such as the tight skin mouse (Tsk1/+) (Menon et al., 2006), bleomycin induced lung injury (Bonniaud et al., 2004; Lasky et al., 1998) and a unilateral ureteral obstruction (UUO) model, which leads to tubulointerstitial fibrosis (Yokoi et al., 2001). The targeted over-expression (See section 1.3.3) or repression of CTGF markedly alters extracellular matrix accumulation and fibrosis, and supports the idea that CTGF is required for the development of fibrosis (Li et al., 2006; Yokoi et al., 2001). The direct contribution by CTGF to fibrosis is far from clear. Delivery of adenoviral CTGF alone to the lungs of normal mice induces only transient fibrosis, suggesting the requirement

of other factors or the prolonged expression for CTGF to promote fibrosis (Bonniaud et al., 2003). Studies by Takehara and colleagues have shown dermal injection of CTGF or TGF- β alone into naïve mice induces only transient granulation tissue, whereas serial injections of CTGF after TGF- β resulted in the development of persistent dermal fibrosis (Chujo et al., 2005; Mori et al., 1999). These studies support the theory that CTGF is required for the maintenance of skin fibrosis, and suggest other factors are required in conjunction with CTGF for the induction of fibrosis.

1.4.9 CTGF and Systemic Sclerosis

CTGF was first reported to be elevated in SSc in 1995 (Igarashi et al., 1995). Several groups have subsequently reported CTGF to be consistently over-expressed in SSc (Igarashi et al., 1995; Shi-wen et al., 2000; Igarashi et al., 1996; Zhou et al., 2005b). This has led to CTGF being considered a cardinal feature of SSc. Elevated in circulation, bronchoalveolar lavage and dermal interstitial fluids of SSc patients, CTGF levels correlate with the extent and severity of fibrosis (Dziadzio et al., 2005b; Sato et al., 2000; Shi-wen et al., 2000). Immuno-histochemical studies examining tissue biopsies have also revealed that the highest levels of CTGF expression are present within the fibrotic lesions, unlike TGF- β where levels are highest in inflammatory skin areas (Querfeld et al., 1999; Igarashi et al., 1995), suggesting CTGF may not only act as a marker of fibrosis but also modulate the fibrogenic response. *In vitro* SSc dermal fibroblasts from involved and uninvolved areas of fibrosis exhibit elevated expression of CTGF compared to controls, the highest levels of CTGF expression being associated with involved dermal fibroblasts (Shi-wen et al., 2000). SSc fibroblasts maintain their 'fibrotic phenotype' in culture indeed *in vitro* studies found CTGF expression to be elevated in both involved and uninvolved compared to control dermal fibroblast (Shi-wen et al., 2000). Thus it is an attractive proposition that dysregulated expression of CTGF predisposes patients to the development of fibrosis.

Genome-wide screening of the Choctaw Indians has identified a significant association in the region 6q23-27, which encompasses the CTGF gene (Zhou et al., 2003). In addition studies suggest a high degree of correlation between a single nucleotide polymorphism in the 5' regulatory region of CTGF and the development of SSc (Dr Carmen Fonseca, personal communication). Indirect genetic evidence of the involvement of CTGF in SSc pathogenesis is provided by a recent study of gene expression in monozygotic and dizygotic twins (Zhou et al., 2005b). This study

demonstrated elevated expression of CTGF in dermal fibroblasts from an SSc patient and their healthy monozygotic twin. Media transfer studies from either twin resulted in elevated CTGF expression in normal dermal fibroblasts suggesting a secreted factor regulates CTGF expression (Zhou et al., 2005b). The autoinductive effect of CTGF may represent a key mechanism by which CTGF expression is persistently elevated in SSc (Riser et al., 2000; Wang et al., 2003). Collectively the elevated expression of CTGF in dermal fibroblasts of uninvolved skin, and from healthy monozygotic twins of SSc patients suggests over-expression of CTGF alone is not pathological. Rather these studies support the theory that further factors are required acting in concert with CTGF promote fibrosis. In addition to the extensively investigated role of CTGF in TGF- β function, a study by Gore-Hyer *et al.* demonstrated that increased responsiveness of SSc fibroblasts to CTGF-mediated collagen synthesis required co-stimulatory activation by an effector, such as insulin (Gore-Hyer et al., 2003). To date the molecular mechanisms that underlie the over-expression of CTGF in SSc, and indeed other pathological conditions, remain unknown. Previous studies have defined several dysregulated signalling pathways in SSc and their functional effects on elevated collagen gene expression (discussed in section 1.2.7.4). Several of the factors which induce collagen gene expression and the signalling pathways by which they mediate this have been implicated in normal regulation of CTGF expression *in vitro*. Clearly, defining the signalling pathways which promote CTGF expression in normal and pathological situations may shed light on the underlying mechanisms by which fibrosis develops and persists in SSc.

Disease	Reference
<i>Fibrotic Pathologies</i>	
Systemic sclerosis	(Igarashi et al., 1995)
Keloids, eosinophilic fasciitis, Dupuytren's	(Igarashi et al., 1996)
Kidney (glomerulosclerosis, tubulointerstitial, Diabetic nephropathy)	(Ito et al., 1998)
Atherosclerosis	(Wahab et al., 2001b)
Liver cirrhosis	(Oemar et al., 1997)
Central nervous system glial scar formation	(Abou-Shady et al., 2000)
Corneal scarring	(Schwab et al., 2000)
Viral- Hepatitis C	(Wunderlich et al., 2000)
Idiopathic Pulmonary	(Paradis et al., 1999)
Asthma	(Pan et al., 2001)
	(Black et al., 2003)
<i>Inflammatory Pathologies</i>	
Inflammatory bowel disease (Crohn's, ulcerative colitis)	(Dammeier et al., 1998b)
<i>Tumorigenesis</i>	
Pancreatic cancer	(Wenger et al., 1999)
Acute lymphoblastic leukaemia	(Vorwerk et al., 2000)
Cartilaginous tumours	(Shakunaga et al., 2000)
Glioblastoma	(Pan et al., 2002)
Mammary carcinoma	(Nakanishi et al., 2000)
Prostate cancer	(Yang et al., 2005)

Table 1.9

Enhanced CTGF expression in human pathologies.

Dysregulated expression of CTGF expression has been reported in a variety of human pathologies.

1.4.10 CTGF as therapeutic target

TGF- β has been regarded as an excellent target for anti-fibrotic therapies. Several studies have shown targeting TGF- β and the downstream signalling pathways abrogates the development of fibrosis in animal models of fibrosis (Nakao et al., 1999; Yamamoto et al., 1999a; de Gouville et al., 2005). However the normal functions of TGF- β in the body, in particular the anti-proliferative effects on immune cells (Moustakas et al., 2002) make chronic administration of any inhibitor that indiscriminately blocks TGF- β activity problematic due to unwanted side effects. Therefore the use of anti-CTGF therapy may be a more attractive and appropriate mechanism of targeting fibrosis.

Recently, antisense oligonucleotide and interference siRNA to CTGF have been used ameliorate fibrosis in several animal models (Yokoi et al., 2004; Li et al., 2006). These molecules have been shown to block TGF- β stimulated collagen synthesis *in vitro* and support the notion that CTGF represents an anti-fibrotic target. In clinical use there are no specific drugs to prevent the over-expression of CTGF, however anecdotal down regulation of CTGF has been noted with the use of drugs with diverse actions in diseases with fibrotic components. In patients with diabetic nephropathy treated with the angiotensin II receptor blocker, Losartan, a reduction in levels of urinary excreted CTGF was noted and was associated with a slower rate of decline in glomerular filtration rate (Andersen et al., 2005). Significantly studies by Stratton *et al.* noted that patients with SSc receiving prostacyclin derivative, Iloprost, as a treatment for severe Raynaud's phenomenon report a reduction in skin tightness, suggesting that this drug inhibits skin fibrosis. Iloprost treatment markedly reduced the level of CTGF in interstitial fluids. Iloprost represses the excessive CTGF expression in SSc fibroblasts through the activation of PKA and the blockade of the Ras/MEK/ERK signalling pathway (Stratton et al., 2001; Stratton et al., 2002).

1.5 Summary

In summary the maintenance of the SSc fibroblast phenotype in culture suggests an autocrine mechanism which promotes, albeit for a limited number of passages in culture, the persistence of the 'fibrogenic' phenotype. CTGF has been implicated in numerous fibrotic disorders and animal models of fibrosis. Further CTGF has been shown to be essential for many of the actions of TGF- β , a potent modulator of fibroblast function in addition to potently inducing CTGF expression. Thus defining the pathological mechanism(s) which result in the persistent over-expression of the functionally relevant gene, CTGF is likely to define key signalling pathways and components in the pathology of SSc.

Chapter 2

Materials and Methods

2.1 Patients, Clinical details and Biopsies

2.1.1 Patients

All the scleroderma patients in this study fulfilled the American College of Rheumatology (ACR) preliminary criteria for disease (1980) and were classified as Diffuse Systemic Sclerosis (SSc) using internationally accepted criteria (LeRoy et al., 1988).

2.1.2 Clinical details

The clinical and laboratory parameters used for the assessment of disease included duration of scleroderma, skin score, internal organ involvement, and serology. The extent of skin involvement in patients with scleroderma was assessed using a modified skin score index (Kahaleh et al., 1986), being higher than 10 in those with Diffuse Systemic Sclerosis (SSc) (LeRoy et al., 1988).

2.1.3 Biopsies

3-6 mm³ punch biopsies from the skin of normal healthy volunteers and diffuse scleroderma skin biopsies were taken from involved skin in the Rheumatology Unit and Department of Surgery at the Royal Free Hospital during the course of these studies. Biopsies were usually obtained from forearm skin on the non-dominant limb. Informed consent and ethical approval was obtained in all cases.

2.2 Cell Culture

2.2.1 Primary dermal fibroblasts

Fibroblasts were obtained from the biopsies by *in vitro* culture as previously described (Abraham et al., 1991). Briefly, dermal biopsies were cut into 1-2 mm³ pieces and placed in sterile plastic dishes or flasks. After 15 min of drying at room temperature the pieces of biopsy were adherent to the tissue culture plastic and then cultured in fibroblast growth medium (FGM) consisting of Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% foetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, 50 µg ml⁻¹

gentamycin and 2.5 $\mu\text{g ml}^{-1}$ amphotericin B. After 2-3 weeks of incubation in a humidified atmosphere of 5% CO_2 in air at 37°C, the fibroblast outgrowths were detached by brief treatment with trypsin (0.125% trypsin in phosphate buffered Saline (PBS), 0.5 mM EDTA) for 3 min at 37°C and re-cultured in FGM, but without gentamycin and amphotericin B. The fibroblast phenotype was confirmed by their morphology in monolayer and fibroblasts on reaching confluence were routinely re-cultured by diluting in a 1:3 ratio. In experiments, fibroblasts were used between passages 2 and 5 unless otherwise stated. All cells were maintained in a humidified atmosphere containing 5% CO_2 in an incubator (Hera Cell240) at 37°C.

2.2.2 Fibroblast lines

Human foreskin fibroblasts (HFF), a generous gift of Dr K Yamada (National Institute of Health) (Cukierman et al., 2001), were grown in FGM medium without gentamycin and amphotericin B, and all experiments were performed between passage 13 and 18. Cells from the mouse fibroblast cell line, NIH/3T3, established from the NIH Swiss mouse embryo (Jainchill et al., 1969) (The European Collection of Cell Cultures), were cultured in DMEM supplemented with 10% calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units ml^{-1} penicillin, 100 $\mu\text{g ml}^{-1}$ streptomycin (N-FGM). Both cell lines, upon reaching 90% confluence were trypsinised and sub-cultured by dividing 1:3.

2.2.3 TGF- β treatment

Human recombinant TGF- β 1 (from R and D Systems) was used at 2 ng ml^{-1} unless otherwise stated. TGF- β was added to confluent fibroblast cultures and incubated for 24 h unless stated otherwise, after which the cell monolayers were washed with fresh media and used immediately in the experiments detailed below.

2.2.4 Transient transfection

Transient transfection of fibroblasts was performed using liposomal formulated, Lipofectamine Plus (Invitrogen) and non-liposomal formulated FuGENE 6 (Roche) transfection reagents. Briefly, fibroblasts were plated at 50-80% confluence in 12-well culture plates in the appropriate growth media prior to the day of experimentation. After 18 h seeded cells were rinsed with DMEM and maintained in 0.5 ml of DMEM containing 0.2% FCS for 4 h.

For transfections with FuGENE 6, serum and antibiotic free DMEM was warmed to 37°C, and 50 µl per well was transferred to a microfuge tube and the appropriate volume of FuGENE 6 per transfection added (See Chapter 3; 3.3.2.3). The tube was mixed by tapping, and incubated for 5 min at room temperature. DNA (500 ng per well) was added and the sample incubated at room temperature for a further 15-30 min. The complex was added drop-wise to its designated well and the plate swirled to ensure even mixing of the complex in all the wells. The cells were returned to the incubator and maintained in a humidified atmosphere containing 5% CO₂ at 37°C. After 8 h, media was replaced with the FGM containing 0.2% FCS.

Transient transfection of fibroblasts with Lipofectamine Plus (Invitrogen) was performed in a similar manner to that of FuGENE 6. Briefly, serum and antibiotic free DMEM was warmed to 37°C, and 50 µl per well was transferred to a microfuge tube, DNA (500 ng per well) and 4 µl of Plus reagent (Invitrogen) was added and the 'pre-complex' sample incubated at room temperature for 15 min. To 50 µl of serum and antibiotic free DMEM, 2 µl lipofectamine (Invitrogen) reagent per transfection (as determined in Chapter 3; 3.3.2.3) was added. The tube was mixed by gentle tapping and added to the 'pre-complex', mixed and incubated at room temperature for a further 15 min. The lipofectamine/DNA complex was added drop-wise to its designated well and the plate swirled to ensure even mixing of the complex in the wells. The cells were returned to the incubator and maintained in a humidified atmosphere containing 5% CO₂ at 37°C. After 8 h, media was replaced with the FGM containing 0.2% FCS and, where appropriate, TGF-β added for a further 24 h. For experiments performed in 6-well culture plates, DNA and transfection reagents were scaled accordingly.

2.2.4.1 Transient transfection of promoter reporter constructs

To study the transcriptional regulation of CTGF, promoter reporter constructs (Figure 2.1) were transfected into both primary, HFF and NIH/3T3 cell lines. The promoter constructs were sub-cloned into the pSEAP basic plasmid (Clontech) containing the truncated form of human placental alkaline phosphatase (SEAP) reporter gene. Promoter constructs linked to the reporter gene firefly luciferase were treated in the same manner. Transfection efficiency was controlled for by co-transfection with the SV40 driven β-Galactosidase reporter gene construct, pSV-β-Galactosidase (Invitrogen). Fibroblasts were transfected as described above (Chapter 2; 2.2.1) with 500 ng per well of reporter and expression vectors unless otherwise stated and 20 ng per

well of LacZ reporter gene. For assays in which the role of trans-acting proteins were tested, 200ng of reporter and 100ng of expression vector encoding the protein of interest were included in the transfection protocol. Using the blank expression vector, pcDNA3.1, DNA was made up to a total of 500 ng per well.

After 24 h in the presence or absence of TGF- β , media was collected. The cell monolayer was washed once with PBS and lysed in 120 μ l of lysis buffer (Tropix). Both media and lysed monolayers were stored at -20°C. Reporter gene activity was determined as below. All cell mono-layers were assessed for cell viability by visual appraisal.

2.2.4.2 Secreted alkaline phosphatase (SEAP) and β -galactosidase reporter gene activity

Media from cells transfected with promoter constructs linked to the SEAP reporter gene was defrosted to room temperature and reporter activity assessed using the Phospha-Light system as per the manufacturer's instructions (Applied Biosystems). Briefly, for each transfection 300 μ l of dilution buffer was added to 100 μ l of media in a microfuge tube and the mixture heated at 65°C for 20 min. 100 μ l of the sample was removed and placed in a white 96 well flat bottom assay plate (Costar). To each well 100 μ l of assay buffer, equilibrated to room temperature, was added and the mixture incubated at room temperature for 5 min. 100 μ l of reaction buffer containing the Chemiluminescent alkaline phosphatase substrate, CSPD (Bronstein et al., 1994) was added and incubated for 20 min at room temperature. Activity was determined in either a Mithras LB940 (Mithras) luminometer, or, for some of the experiments, a single read luminometer TD-20/20 (Tropix) was used. Luminescence, measured in relative light units (RLU) was determined for 1 sec and the background level of RLU in media from un-transfected (control) cells subtracted.

To normalise samples for differences in transfection efficiency, β -galactosidase activity encoded for by the co-transfected gene construct pSV- β -Galactosidase, was determined using the Galacto-Light Plus system (Applied Biosystems). Briefly, to 10 μ l of the lysed mono-layers 70 μ l of reaction buffer, containing the galactosidase substrate galacton, was added and the mixture incubated for 30-60 min at room temperature. To each sample 100 μ l of accelerator was added and the luminescence determined for 0.2 sec.

SEAP RLU values determined twice for each sample were normalized to galactosidase RLU.

2.2.4.3 Determination of Luciferase and β -galactosidase reporter gene activity

Expression of luciferase and β -galactosidase was determined using the Dual light system as per the manufacturer's instructions (Applied Biosystems). Briefly, to 10 μ l of each lysed cell mono-layer 25 μ l of Buffer A, equilibrated to room temperature, was added and the mixture incubated at room temperature. After 5 min, room temperature equilibrated Buffer B containing the galactosidase substrate Glacton-Plus (1:100 Glacton-plus to Buffer B) was added and luciferase signal read for 2 sec as described in 2.2.4.2. After a further incubation of 30 min, 100 μ l of Accelerator-II was added to each well and galactosidase activity (RLU) from the co-transfected pSV- β -Galactosidase constructs determined for 0.2 sec. All luciferase RLU values were normalized to galactosidase RLU in order to control for transfection efficiencies.

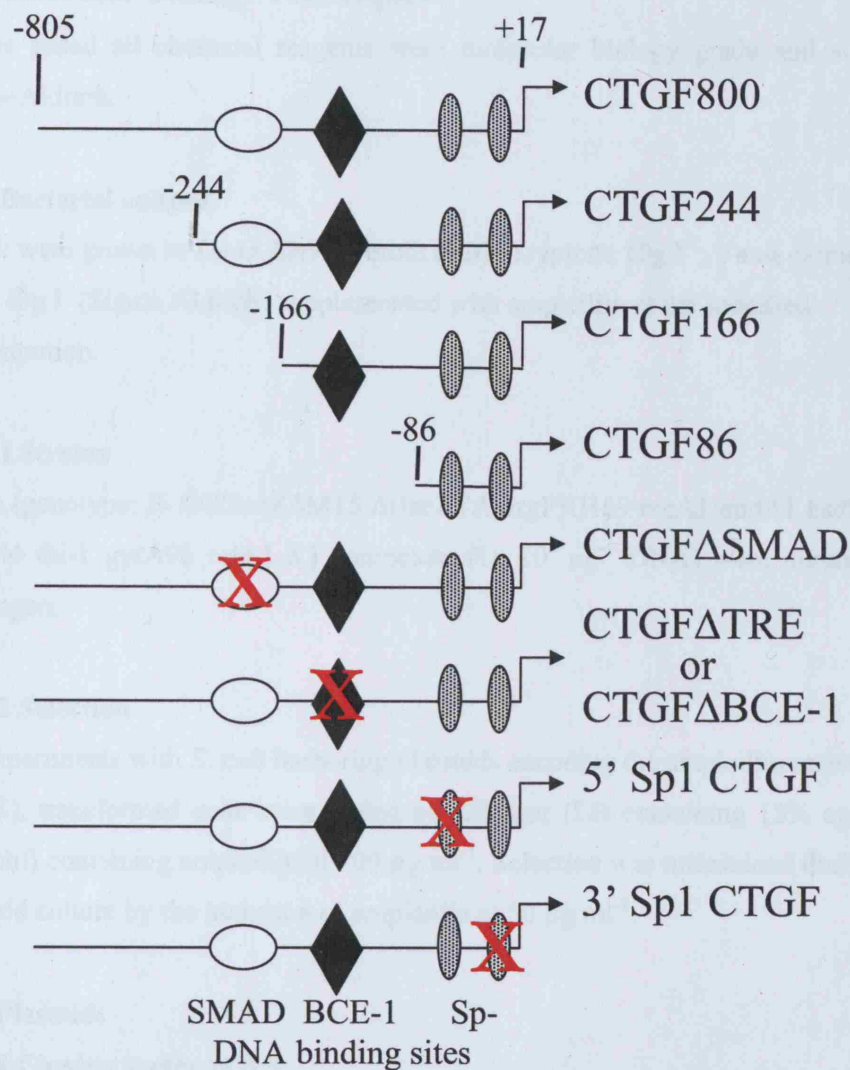


Figure 2.1

Schematic of CTGF promoter deletion and point mutations constructs.

The CTGF promoter (-800 to +17bp) was linked to the reporter gene secreted alkaline phosphatase (SEAP). Deletion series were generated containing 244bp, 166bp and 86bp of the promoter. The putative transcription factor binding sites Smad and Sp1-like and the previously characterised TGF- β response element, T β RE (BCE-1), is as shown. Point mutations are denoted by **X**.

2.3 Molecular Biology Techniques

Unless stated all chemical reagents were molecular biology grade and supplied by Sigma-Aldrich.

2.3.1 Bacterial culture

E. coli were grown in *Luria-Bertani* broth (LB) (Tryptone 10g l⁻¹; Yeast extract 5g l⁻¹; NaCl 10g l⁻¹ (Sigma Aldrich) supplemented with ampicillin at the specified concentration.

2.3.1.1 Strains

DH5 α (genotype: F- Φ 80lacZ Δ M15 Δ (lacZYA-argF)U169 recA1 endA1 hsdR17 PhoA SupE44 thi-1 gyrA96 relA1 λ) competent (1x 10⁸ μ g⁻¹ DNA) were purchased from Invitrogen.

2.3.1.2 Selection

For experiments with *E. coli* harboring plasmids encoding the ampicillin resistance gene (amp^R), transformed cells were plated on LB-agar (LB containing 15% agar (Sigma Aldrich)) containing ampicillin at 100 μ g ml⁻¹. Selection was maintained during growth in liquid culture by the inclusion of ampicillin at 50 μ g ml⁻¹.

2.3.2 Plasmids

2.3.2.1 Cloning vector pGL3

The pGL3 basic cloning vector (Promega) is derived from pUC19, with ampicillin resistance and a ColE1 origin of replication for propagation in *E. coli*. The synthetic multiple cloning site (MCS) contains 8 unique restriction sites. The plasmid also harbours a modified coding region for firefly (*Photinus Pyralis*) luciferase for the quantitative analysis of cis-acting factors in transfected eukaryotic cells allowing. (Groskreutz DJ, 2005).

2.3.2.2 Expression Vectors

The following CMV expression plasmids were used during the course of the studies:

Plasmid	Gene Encoded	Function	Source	Reference
pFlag hSmad2	Smad2	OE	J. Massagué	(Hata et al., 1997)
pFlag hSmad3	Smad3	OE	J. Massagué	
pFlag hSmad4	Smad4	OE	J. Massagué	(Hata et al., 1997)
pFlag-mSmad6	Smad6	OE	J. Massagué	(Hata et al., 1998)
pFlag-mSmad7	Smad7	OE	J. Massagué	
pFAST-1	FAST-1	OE	A. Roberts	(Felici et al., 2003)
pSp1	Sp1	OE	D Kardassis	(Birnbaum et al., 1995)
pSp3	Sp3	OE	G Suske	(Ghayor et al., 2001)
pcDNA3.1	None	TC	Invitrogen	
pSV- β -Gal	LacZ	OE	Promega	

Table 2.1
Expression vectors.

Constitutively over-expressed genes (OE) were driven by the cyclomeglovirus (CMV) promoter. LacZ- β -Galactosidase gene. Transfection control (TC) background vector of OE constructs.

2.3.2.3 Promoter reporter constructs

The following reporter constructs were used throughout these studies

Plasmid	Details	Reporter Gene	Gift of/ Generated by	Reference
CTGF800	800bp CTGF (-804 +17bp)	SEAP	Dr A Leask	(Holmes et al., 2001)
CTGF244	244bp CTGF (-244 +17bp)	SEAP	Dr A Leask	As above
CTGF166	166bp CTGF (-166 +17bp)	SEAP	Dr A Leask	As above
CTGF86	86bp CTGF (-86 +17bp)	SEAP	Dr A Leask	As above
CTGFΔBCE1	PM in TβRE	SEAP	Dr A Leask	As above
CTGF ΔSMAD	PM in Smad	SEAP	Dr A Leask	As above
5'Sp1 CTGF	PM in 5' Sp1	SEAP	Dr A Leask	(Holmes et al., 2003)
3' Sp1 CTGF	PM in 3' Sp1	SEAP	Dr A Leask	As above
pSEAP Basic	Control vector	SEAP	Invitrogen	
pCAGA	Smad3	Luciferase	Dr A. Roberts	(Felici et al., 2003)
ARE	Smad2	Luciferase	Dr A. Roberts	As above
pGL3	Control vector	Luciferase	Promega	
pGAG6 pGAG	Sp1 reporter Control vector	Luciferase	Dr A Kudlow	(Kumar and Butler, 1998)

Table 2.2.

Promoter/reporter constructs.

CTGF promoter deletion constructs and point mutations (Figure 2.1) were generated in collaboration with by Dr Andrew Leask. PM denotes point mutations in the specified transcription factor binding sites in the context of the -804 to +17 bp of the CTGF promoter/reporter construct (Figure 2.1).

2.3.3 Transformation of competent bacteria with plasmid DNA

Frozen competent cells (Invitrogen) of the *E. coli* strain DH5 α were placed on ice until thawed. Plasmid DNA was added to pre-chilled microfuge tubes containing 100 μ l of competent cells, gently mixed and left on ice. After 30 min the DNA/competent cells were heat shocked by placing the microfuge tubes in a water bath at 42°C for 60 sec, and subsequently transferred back onto ice for a further 3 min. 900 μ l of LB medium was added and incubated for 45 min at 37°C with agitation. The bacterial cells were then pelleted by centrifugation (Heraeus Biofuge Fresco) at 12,000 rpm per min (rpm) for 30 sec and resuspended in 50 μ l of LB media and plated onto selection plates. Plates were dried then inverted and incubated at 37°C overnight (Cohen et al., 1972).

2.3.4 Isolation of plasmid DNA

2.3.4.1 Mini-plasmid preps

Individual colonies were picked from plates and inoculated into 3 ml of LB Amp broth and the plasmid DNA isolated using an alkali lysis procedure. Briefly, 1 ml of medium was transferred to a microfuge tube and the bacteria were pelleted by centrifugation at 13,000 rpm for 1 min and resuspended in 300 μ l of resuspension buffer (50mM Tris.Cl, pH 8.0; 10 mM EDTA; 100 μ g ml⁻¹ RNase A). The cells were lysed by the addition of an equal volume of lysis buffer (200 mM NaOH; 1% SDS (w/v)) and the solution mixed gently and incubated at room temperature until the solution cleared, whereupon an equal volume of neutralization buffer (3 M potassium acetate, pH 5.5) was added and the solution agitated strongly. The precipitate of cell debris, chromosomal DNA and SDS was pelleted by centrifugation at 13,000 rpm for 15 min at 4°C. The supernatant was removed, 0.7 volumes of propan-2-ol added and the plasmid DNA precipitate pelleted by centrifuged at 13,000 rpm for 30 min at 4°C. The pellet was washed with 80% EtOH air dried, and resuspended in 50 μ l of T.E. (10 mM Tris-HCl (pH8.0) 0.1 mM EDTA). All plasmids were validated by diagnostic restriction digestion and agarose gel electrophoresis as described in 2.3.5 and 2.3.6.

2.3.4.2 Maxi-plasmid preps

100 ml of LB containing 100 $\mu\text{g ml}^{-1}$ Ampicillin were inoculated with 100 μl of mini-prep culture and grown with agitation over night (as above). Plasmid DNA was isolated based on a modified alkaline lysis (Birnboim and Doly, 1979) procedure followed by binding of plasmid DNA to an anion-exchange resin, as per the manufacturer's instructions (QIAGEN, UK). In short, the overnight bacterial culture was pelleted by centrifugation at 3000 rpm for 10 min, and resuspended in 20 ml of resuspension buffer. The cells were lysed by the addition of an equal volume of lysis buffer, and the solution mixed gently at room temperature until the solution cleared, whereupon an equal volume of neutralization buffer was added and the solution agitated strongly. The precipitate of cell debris, chromosomal DNA and SDS was pelleted by centrifugation at 10,000 rpm for 15 min at 4°C. The supernatant was removed and passed over the QIAGEN Anion-Exchange resin, the bound plasmid DNA washed with low salt buffer (1 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v) and finally eluted in 5 ml of elution buffer (1.6 M NaCl; 50 mM MOPS, pH 8.5; 15% isopropanol (v/v)). The eluted DNA was precipitated with the addition of 0.7 volumes propan-2-ol, and the DNA pellet washed in 80% EtOH and air dried. The DNA pellet was resuspended in appropriate volume T.E. and the concentration was determined from the absorbance at A_{260} of the preparation, assuming that a DNA solution of 50 $\mu\text{g ml}^{-1}$ in water gives a value of $A_{260} = 1$ using a spectrophotometer (Hitachi U2001). Plasmid DNA was stored at 1 mg ml^{-1} in TE, at 4°C (short term) or -20°C (long term).

2.3.5 Restriction enzyme digests

Restriction enzymes were used according to the manufacturer's instructions (Promega). In general, 5 μg of plasmid of DNA was cut with 50 units of restriction enzyme for 18 h at 37°C in the presence of 150 $\mu\text{g ml}^{-1}$ bovine serum albumin (BSA) to stabilize the enzyme. Where multiple digests were to be performed, the buffer conditions were selected to be compatible with both enzymes.

2.3.6 Agarose gel electrophoresis of DNA

Gels were prepared by boiling the appropriate mass of low melting point or general purpose agarose in 1x Tris-Acetate-EDTA (TAE; 242 g Tris base, 57.1 ml Acetic acid and 100ml 0.5M EDTA to a total volume of 1 liter with ddH₂O and adjusted to pH 8.5) cooling to 50°C before adding Ethidium Bromide (EtBr) at 1 $\mu\text{g ml}^{-1}$ and then setting this in the chosen gel former; "mini" gels (6.5 x 10 x 1 cm, 15 sample wells). All gels

were run in 1xTAE; at 10 volts cm^{-1} . DNA fragments were satisfactorily resolved on 1-2% agarose gels.

2.3.7 Detection and isolation of DNA fragments

Ethidium bromide intercalates DNA, and in this state fluoresces when illuminated by ultraviolet (UV) light. DNA was visualized by illuminating the gel with short wave UV light on a transilluminator (Stratagene). When DNA was to be recovered from the gel, a hand-held long wavelength lamp was used to avoid damage to the DNA. DNA fragments, after restriction endonuclease digestion, were excised from the agarose gel and the slice containing the DNA fragment purified as per manufacturer's instructions using QIAquick gel extraction (Qiagen).

2.3.8 Analysis of fibroblast mRNA by Northern Hybridisation

2.3.8.1 RNA extraction from fibroblast monolayers

RNA was extracted from fibroblast monolayers using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Briefly, culture medium was aspirated and cells were gently washed with sterile PBS at 4°C. The PBS was removed and 1 ml of TRIzol per 10^6 cells was added to each dish and incubated at room temperature for 10 min to lyse the cells. The lysate was pipetted several times then 1ml was transferred to a 1.5ml RNase free microfuge tube. Upon transfer 0.2 ml of chloroform was added per 1ml. Tubes were shaken vigorously for 15 sec and then incubated at room temperature for a further 2 min. The samples were then centrifuged at 13,000 rpm at 4°C for 15 min. After centrifugation the aqueous phase was transferred to a fresh 1.5ml tube and an equal volume of isopropanol was added to precipitate the RNA. After 5 min incubation the sample was centrifuged at 12,000xg at 4°C for 30 min. The pellet was washed in 80% ethanol and resuspended in an appropriate volume of diethylpyrocarbonate (DEPC) treated water. To confirm integrity a sample of the RNA was subjected to gel electrophoresis.

RNA concentration and purity were measured spectrophotometrically (Hitachi U2001) by determining the absorbance of a diluted aliquot (of 4 μl of sample with 796 μl DEPC treated water in a quartz cuvette) at 260nm. The RNA concentration was determined based on the absorbance co-efficient of an $\text{OD}_{260\text{nm}}=1$ equivalent to a RNA solution of 40 $\mu\text{g ml}^{-1}$. The absorbance of the solution at 280nm was also measured and a ratio of $\text{OD } 260\text{nm}:280\text{nm}$ was used as an index of purity. Samples with a ratio of less than 1.6

were re-extracted. Sample integrity was confirmed by agarose gel electrophoresis as described in 2.2.5.8. Generally the yield for 10^6 fibroblasts was 15-25 μg of total RNA.

2.3.8.2 Northern blotting

2.3.8.2.1 Electrophoresis of RNA and transfer to Hybond membrane

RNA samples were electrophoretically separated on a 1% (w/v) denaturing formaldehyde-agarose gel. This was prepared by dissolving 1g of molecular biology grade agarose in 66 ml DEPC treated water, adding 20 ml of 5X gel running buffer (0.1M MOPS, 40mM sodium acetate, 5mM EDTA, pH7.0) and then 14 ml 2.2 M formaldehyde to a final gel concentration of 2.2M. Samples were prepared as follows: 10 μg of RNA was added to a microfuge tube and DEPC-treated water added to a final volume of 4.5 μl to which formaldehyde (3.5 μl), formamide (10 μl) and 5X running buffer (2 μl) were added. This sample mixture was incubated at 65°C for 15 min, and kept on ice before loading. Prior to loading, 2 μl of 6X gel loading buffer (50% glycerol, 1mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol) was added to each sample. The gel was pre-run for 5 min at 5V cm^{-1} then samples were loaded and electrophoresis performed for 1-2 h at 3-4V cm^{-1} .

The agarose gel was rinsed in DEPC-treated water to remove the formaldehyde and soaked in 20X sodium chloride-sodium citrate (SSC) buffer (3.0 M NaCl, 0.3 M Na-citrate) for 45 min. The gel was placed on blotting paper, pre-soaked in 20X SSC, hybond membrane placed on top of the gel and a piece of pre-soaked blotting paper placed over the membrane. Multiple (8-10) dry blotting papers were placed on top of the soaked layer, a glass plate was placed over the dry layers and transfer performed for 4-12 h at room temperature. After transfer the RNA was UV cross linked to the hybond membrane (15 sec at 254nm) using a UV stratalinker (Stratagene)

2.3.8.2.2 Radiolabelling of cDNA probes

CTGF and GAPDH cDNA fragments were a kind gift of Dr Sarah Howat (King's College London). Labelling of DNA fragments was performed using a Megaprime labelling kit (Amersham Life Sciences) according to the manufacturer's instructions. In brief, approximately 25 ng of template DNA was added to 5 μl of random primers and boiled for 5 min to denature the DNA. The tube was placed on ice and 10 μl labeling buffer, 5 μl of αP^{32} dATP (250 uCi; 9.25 MBq). and 2 μl of DNA polymerase (Promega) were added. The mixture was then incubated for 30 min at 37°C. The

labelling reaction was stopped by addition of 50 μ l of 4M ammonium acetate (pH4.5) and the probe purified from unincorporated radiolabel using a microspin G50 column (Amersham Life Sciences) as per manufacturer's instructions. Using this protocol, probes were labelled to a specific activity of at least 10^9 dpm μ g⁻¹.

2.3.8.2.3 Hybridisation

The hybridisation bottle was half-filled with 2X SSC, and the membrane applied to the inside wall of the bottle. The SSC was poured off and replaced with 5ml Rapid-hyb (Invitrogen) buffer. The membrane was pre-hybridised in a Hybaid oven at 65°C for 15 min. Prior to the addition of the labelled probe to the hybridisation buffer, cDNA was denatured by boiling for 5 min. The labelled probe was added and hybridisation performed at 65°C for 2 h. The membrane was washed twice with 2X SSC at room temperature, twice with 1X SSC at 65°C and then rinsed in 2 X SSC.

2.3.8.2.4 Autoradiography and quantitation

The membrane was placed against X-Ray film, and left at -70 °C between 12-72 h. X-ray films were digitised using a digital camera (UVP v1.0) and UVP Grab software (Synoptics, UK).

2.3.8.3 Quantitative real-time PCR

2.3.8.3.1 DNase treatment

Genomic DNA was removed from the total RNA isolated by DNase digestion to ensure sample purity. Briefly, 10-20 μ g ml^{-1} of total RNA isolated as described in 2.2.5.7.1 was re-suspended to a final volume of 100 μ l containing 10-20 units of RQ1, RNase-Free DNase (Promega) and 40 mM Tris-HCl (pH8.0), 10 mM MgSO_4 and 1 mM CaCl_2 in DEPC-treated water and the samples were incubated at 37 °C for 30 min. Samples were phenol:chloroform extracted to remove DNase and the RNA in the aqueous layer precipitated as described previously. RNA was resuspended and concentration adjusted to 1 μ g μ l⁻¹.

2.3.8.3.2 First Strand cDNA synthesis

First strand cDNA was generated as per the manufacturer's instructions (Promega). Briefly, total RNA (1 µg) in a total volume of 20 µl containing an oligonucleotide (dT₁₈) and random decamers (dN₁₀) were denatured at 65°C for 5 min to remove secondary structure. Samples were cooled on ice for 5 min. cDNA was reverse transcribed for 1 h at 37 °C using M-MLV reverse transcriptase (Promega) in a total volume of 30 µl. The cDNA was diluted to 100 µl with DEPC-treated water and 1 µl was used for all subsequent reactions.

2.3.8.3.3 Real time PCR

Quantitative real-time PCR experiments were performed by Dr Sarah Howat (Kings College London) using QuantiTect SYBR Green system (Qiagen) and performed in a ABI Prism 7700 system (Applied Biosystems, Warrington, UK) as described previously (Xu et al., 2004). Each reaction contained 10 ng of reverse transcribed RNA in a total volume of 12 µl. Primers were as described in Table 2.3. Triplicate samples were run, transcripts were measured in picograms, and expression values were standardized to values obtained with control 28 S RNA primers and expressed as fold changes.

Primer Name	Forward Primer	Reverse Primer	Amplicon Size (bp)	Reference
CTGF _m	CCC ACA CAA GGG CCT CTT C	CCA TCT TTG GCA GTG CAC AC	186	(Higgins et al., 2003)
CTGF _h	CTC GCG GCT TAC CGA CTG	GCA CTT GAA CTC CAC CGG	223	(Xu et al., 2004)
28S rRNA	TTG AAA ATC CGG GGG AGA G	ACA TTG TTC CAA CAT GCC AG	185	(Xu et al., 2004)
COL1A2 _h	TGG TGA AAC TGG TCC TTC TG	TTA AGC CAG GAA GAC CTC TGG	133	
PAI-1 _h	AGA TCG AGG TGA ACG AGA GAG TGG CAC G	TTT GTC CCA GAT GAA GGC GTC TTT CC	199	

Table 2.3

Real time PCR primers.

Single stranded oligonucleotides (5'-3') were synthesised (MWG Biotech). Primers denoted with the subscript 'h' were designed against the human whereas those denoted by 'm' preferentially amplified the mouse mRNA transcript.

2.4 Protein extraction

2.4.1 Preparation of total protein samples

Fibroblasts were seeded (2.5×10^5 cells per well) in six-well tissue culture plates 18 h prior to experimentation in FGM. The medium was replaced with DMEM containing 0.2% FCS for 24 h. Cell monolayers were washed twice in serum-free medium and replaced with DMEM containing 0.2% FCS for 24 h in the presence or absence of TGF- β 1 for a further 24 h. For experiments where collagen type I was to be assessed, 1 ml of media was removed and 300 μ l saturated ammonium sulfate added, and the sample rocked at 4°C overnight. The next day the samples were centrifuged at full speed (13,000 rpm) for 30 min at 4°C, and the pellet was resuspended 50 μ l of RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4 mM EDTA 1 mM PMSF; 1% NP-40; 1% Sodium deoxycholic acid; 0.1% SDS; 1% protease inhibitor cocktail inhibitor (Boehringer Mannheim)). For all cell-associated proteins, cell monolayers were washed twice in ice cold PBS and lysed in 150 μ l RIPA buffer. The lysed cells were scraped and DNA sheared by repeated passage of the sample through a 23-gauge needle.

2.4.2 Determining protein concentration

Total protein content of tissue culture supernatants, nuclear extracts and cell lysates were determined using the Bio-Rad protein micro-assay. Standard concentrations of bovine serum albumin (BSA) diluted in culture medium or lysis buffer, depending on the nature of the samples being tested, were used to calibrate the assay and confirm its reliability in the concentration range being measured. The assay was performed according to manufacturer's instructions (Bio-Rad, Munchen, Germany). Briefly, protein standard was prepared by serial dilutions containing from 1 to 25 μ g ml⁻¹ BSA. 200 μ l of standards and diluted samples were added to replicate wells of a 96 well flat bottomed plate. Then, 50 μ l of dye reagent concentrate was added to each well. After mixing, the test plate was incubated for 30 min at room temperature. Absorbance was measured on an automatic plate reader at a wavelength of 595 nm. The absorbance for standards was plotted against protein concentration to give a standard curve and linear regression analysis used to determine the protein concentration of the test samples.

2.4.3 Protein Expression- Western blot analysis

2.4.3.1 Electrophoresis and transfer of protein samples for Western blotting

Protein samples had an appropriate volume of 6x Laemmli sample buffer (0.2 M Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 0.004% bromophenol blue) and 1 μ l of 14.2 M β -mercaptoethanol, added and were heat denatured at 100°C for 3 min. The proteins are resolved by passing a current across a polyacrylamide gel, separating protein based on size. 20 μ l, containing 10-20 μ g of protein were run upon a ready-cast 12% Tris-Glycine gel (Invitrogen) alongside a broad-range protein marker (New England Biotech) at 100V, until the dye front had reached the bottom of the gel (approx 1 ½ h) in 1x Tris-Glycine Running Buffer (Invitrogen). This was carried out within an Electrophoresis tank (Invitrogen), ensuring that the running buffer (25 mM Tris Base, 192 mM Glycine, 0.1 % SDS, pH 8.3) completely covered the gel at all times.

Each gel was removed from the casing and placed within a transfer set-up, using chromatography paper, Hybond C+ membrane (Amersham), and sponge filters. All these components had previously been soaked in 1x transfer buffer (25 mM Tris Base, 192 mM Glycine, 20 % Methanol). The transfer set-up was then carefully placed within a transfer module (Invitrogen) and placed in an electrophoresis tank (Invitrogen), completely submerged in 1x transfer buffer. Overheating during transfer was prevented, by surrounding the transfer module with cold water. The transfer was then allowed to proceed at 30V for approximately 1½ h. After this time, the blue dye from the protein markers could be clearly seen transferred onto the Hybond™ membrane, indicating that transfer had occurred. Each membrane was removed from the transfer apparatus and protein transfer confirmed by staining for 1 min in Ponceaus solution (0.1% Ponceaus S (w/v), 5% acetic acid (v/v)). Membranes were washed in 0.5% PBS-Tween for three washes of 10 min, with constant agitation.

For detection of type I collagen samples were resolved on a 6% Tris-Glycine gel (Invitrogen) as described above.

2.4.3.2 Immunoblotting

Immunoblotting was performed by incubating the membranes in PBS-Tween (0.01% (v/v) Tween 20) with 5% non-fat dry milk at 4°C overnight to block non-specific antibody binding. The blocking solution was removed and the primary antibody added at the appropriate concentration (Table 2.4) to PBS-Tween (0.01% (v/v) Tween 20) with 5% non-fat dry milk and incubated for 2 h at room temperature. The membranes were then washed in PBS-Tween, three times for 15 min and then incubated with the appropriate peroxidase-conjugated affinity purified secondary antibody (Table 2.5) in PBS-Tween containing 5% milk at room temperature for 1 h. The membranes were washed and the blot was developed using Luminol/enhancer solution plus stable peroxide solution (Pierce, Rockford, Illinois, USA). The membrane was exposed to ECL Hyper film (Amersham, UK) for between 30 sec and 5 min and the film developed.

For Western blots for phosphorylated proteins PBS was replaced with Tris-Buffered Saline (TBS; 0.05M Tris Base, 0.9% NaCl, pH 7.6).

Membranes were re-probed using the Western blot stripping buffer, Restore (Pierce) as per manufactures instructions. Briefly, membranes were washed in TBS-Tween, two times for 15 min and then incubated with a sufficient volume of Restore to ensure the membrane was completely covered. Membranes were incubated at 37 °C for 5-15 min and removed and washed two times in TBS-Tween. Membranes were incubated with Luminol/enhancer solution plus stable peroxide solution (Pierce, Rockford, Illinois, USA) and removal of antibodies was confirmed by exposure of membranes to ECL Hyper film (Amersham, UK).

2.4.4 Antibodies

Primary (Table 2.4) and peroxidase conjugated secondary (Table 2.5) antibodies were used at the concentrations indicated.

Antibody	Antibody Species	Company	Dilutions Used	Catalogue Number
Smad2	Goat Poly IgG	Santa Cruz	1:1000	sc-6200
Smad3	Rabbit Poly IgG	Santa Cruz	1:1000	sc-8332
Smad4	Rabbit Poly IgG	Santa Cruz	1:1000	sc-7966
Smad7	Rabbit Poly IgG	Santa Cruz	1:500	sc-7004
Sp1	Goat Poly IgG	Santa Cruz D	1:1000	sc-59
Sp3	Rabbit Poly IgG	Santa Cruz	1:1000	sc-644
ERK1/2	Rabbit Mono IgG	Cell signaling	1:1000	9102
Phospho-ERK1/2	Mouse Mono IgG	Cell signaling	1:1000	4377
JNK	Goat Poly IgG	Santa Cruz	1:1000	sc-57
Phospho-JNK1, 2, 3	Rabbit Poly IgG	Santa Cruz	1:1000	sc-6254
PAI-1	Rabbit Mono IgG	Santa Cruz	1:1000	sc-8979
p38	Mouse Mono IgG	Cell signaling	1:1000	9212
Phospho-p38	Goat Poly IgG	Cell signaling	1:1000	9913
Type I Collagen	Rabbit Poly IgG	Novotec	1:2000	20151
GAPDH	Mouse Mono IgG	Abcam	1:10000	AB8245
Actin	Rabbit Poly IgG	SIGMA	1:5000	A2066
CTGF	Rabbit Mono IgG	Fibrogen	1:5000	pAB2
CTGF-h	Human Mono IgG	Fibrogen	1:2000	FBN-19
CTGF	Goat Poly IgG	Santa Cruz	1:1000	sc-14939

Table 2.4
Primary antibodies and concentrations used for Western blotting.

Antibody	Concentration Used	Catalogue Number
Rabbit anti-Human IgG (H+L) - HRP	1:5000	62-7120
Rabbit anti-Mouse IgG (H+L) – HRP	1:5000	62-6520
Rabbit anti-Rabbit IgG (H+L) – HRP	1:5000	62-6120
Rabbit anti-Goat IgG (H+L) - HRP	1:5000	81-1620

Table 2.5
Secondary antibodies used for Western blotting.
 All antibodies were affinity purified and supplied by Invitrogen.

2.5 Electrophoretic Mobility Shift Analysis

Transcription factor interactions with DNA were determined by Electrophoretic Mobility Shift Assays (EMSA) separating protein/labeled DNA complexes according to their sizes on native polyacrylamide gels. Competition unlabelled consensus oligonucleotides, and targeting the DNA/Protein complex with specific antibodies (super-shifting) were used to determine the specificity of factors binding to DNA regions of interest.

2.5.1 Preparation of nuclear extracts

Following 24 h of serum starvation nuclear protein extracts were prepared from NIH-3T3, HFF, primary control or SSc fibroblast cells or NIH-3T3 cells treated with TGF- β 1 (2 ng ml⁻¹). Nuclear protein extracts were prepared as described previously (Kemler et al., 1989). Fibroblasts grown to confluence in either 3 wells of a 6-well plate or a 10cm petri dishes, between 0.4–1 X 10⁶ cells, were washed in TBS and the monolayers detached by scraping. The cells were pelleted by centrifugation at 1000 rpm for 5 mins. The pelleted cells were resuspended in 400 μ l ice cold buffer A (10 mM HEPES pH7.9; 10 mM KCl; 0.1mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF; 3 μ l of

protease inhibitor cocktail (Boehringer Mannheim) by gentle pipetting and transferred to a fresh tube. The cells were placed on ice and allowed to swell for 15 min, after which 25 μ l of 10% Nonidet NP-40 (Fluka) was added and the cells vortexed. The homogenates were centrifugated for 30 sec at 13,000 rpm, the supernatant discarded and nuclear pellet resuspended in 50 μ l ice cold buffer C (20 mM HEPES pH7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF; 1 μ l of protease inhibitor cocktail) and the tube vigorously rocked at 4°C for 15 min. The nuclear extract was centrifuged for 5 min at 13,000 rpm, and the supernatant snap frozen and stored at -70°C. Protein concentrations were determined using the Bio-Rad protein assay as previously described.

2.5.2 Nuclear protein/DNA binding

Consensus double stranded DNA probes were obtained from Promega. Sense (5'-3') and Anti-Sense (3'-5') single stranded oligonucleotides were synthesised (MWG Biotech) to regions of the CTGF promoter and annealed. Briefly, forward and reverse oligonucleotides were combined at 200 pmol μ l⁻¹ in annealing buffer (5mM MgCl₂, 1mM KCl, 15mM NaCl) at a final volume of 100 μ l and boiled for 5 min. The oligonucleotides were cooled to room temperature slowly for 3 hrs. The annealed oligonucleotides were then ethanol precipitated and resuspended at 2 pmol μ l⁻¹ final concentration. Oligonucleotide probes were end-labelled with γ -³²P (250 uCi; 9.25 MBq), in a reaction mixture containing; 2 pmol of double stranded oligonucleotides, 4 μ l [γ -³²P] dATP, 1 μ l T4 Polynucleotide Kinase (Promega) and 2 μ l 10X reaction buffer (700mM Tris-HCl (pH 7.6 at 25°C), 100mM MgCl₂, 50mM DTT) in a total volume of 20 μ l, and incubated at 37°C for 30 min. The probe was purified from unincorporated radiolabel using a microspin G50 column (Amersham Life Sciences) as per manufacturer's instructions.

Protein/DNA binding assays were carried out at 30°C and consisted of 1-5 μ g of nuclear extract and 10 fmoles of probe (1-5 x 10⁴ cpm), in binding buffer (20 mM HEPES (pH7.9), 10% Glycerol, 40 mM KCl, 0.1% NP-40, 0.5 mM EDTA, 0.5 mM DTT, 1 mM PMSF) and 50 ng non-specific competitor poly dI-dC (Roche) in a final volume of 15 μ l. Cold competitors were added at a 100x molar excess relative to labeled probe. For super-shifts, nuclear extracts were incubated with 2 μ l of 2 μ g ml⁻¹ of the indicated super-shift grade antibody for 10 min at 30°C prior to the addition of probe.

Antibody	Catalogue Number
Smad 2	sc-6200x
Smad 3	sc-8332x
Smad 4	sc-7966x

Table 2.6

Supershift antibodies used for EMSA.

All super-shift antibodies were supplied by Santa Cruz at a concentration of 2 mg ml⁻¹.

2.5.3 Resolving Protein/DNA complexes

Samples were resolved on non-denaturing acrylamide gels. Briefly, 6% acrylamide gels (Invitrogen), were pre-run for 1 hr in 0.5x Tris buffered EDTA (TBE; Tris Base 108 g, Boric Acid 55g; 0.5M EDTA 20 mL, ddH₂O to 1.0 L) chilled to 4°C. Samples containing 2 µl of gel loading buffer (250mM Tris-HCl (pH7.5); 40% Glycerol) , were run alongside one lane containing gel loading dye supplemented with 0.2% bromophenol blue (SIGMA) at 100 volts until the dye front had reached the bottom of the gel (approx 1 ½ h). Gels were dried at 80°C (BioRad Gel Dryer and Vacuum pump) and complexes visualized by auto-radiography (BioMaxMR film, Sigma, UK).

2.6 Semi-Quantification of protein expression

All semi-quantitative densitometry analysis was performed using scion software (www.scioncorp.com). ECL Hyper film were scanned (Epsom 1640 Scanner) as 16-bit BMP files and densitometric values for proteins of interest normalized to actin or GAPDH expression.

2.7 Statistical Analysis

The data are presented as means \pm standard error of the mean (SEM), or standard deviation (SD), for 6 replicates, or are representative of experiments performed in triplicate in replicate experiments. Statistical analysis was performed by the Student's unpaired t-test. P values less than 0.05 were considered statistically significant.

Chapter 3:

TGF- β regulation of CTGF in normal fibroblasts

3.1 Introduction

The pathological over-expression of CCN2 in a host of fibrotic conditions has led to significant interest in the regulation of this gene in mesenchymal cells. Identified as a secreted factor produced by human endothelial cells (Bradham et al., 1991), CTGF expression in mesenchymal cells is normally tightly regulated primarily at the level of gene transcription (Brunner et al., 1991; Almendral et al., 1988). In human dermal fibroblasts *in vivo* and also when cultured *in vitro* CTGF is expressed at low levels (Igarashi et al., 1996; Shi-wen et al., 2000; Grotendorst et al., 1996; Igarashi et al., 1993). In the mid 1990s Grotendorst *et. al.* demonstrated TGF- β up-regulated CTGF via a region in the CTGF promoter termed the TGF- β RE or TRE (Grotendorst et al., 1996). The absence of this element rendered the CTGF promoter insensitive to TGF- β 1 activation in several fibroblast cell types, including murine NIH/3T3 fibroblasts (Grotendorst et al., 1996; Igarashi et al., 1993). The sequence of this element, GTGTCAAGGGGTC, does not contain any sequence homology to known TGF- β control elements in other genes (Greenwel et al., 1997; Hocevar et al., 1999). This led to it being considered a novel TGF- β response element. Until the mid-1990s TGF- β was known to activate and induce genes via several generic signalling pathways including p38 and JNK MAPKs (Sato et al., 2002; Hocevar et al., 1999). The subsequent identification of the TGF- β specific signalling pathway, which utilises the Smad family of proteins (Lagna et al., 1996), has led to the re-evaluation of the mechanism by which TGF- β impacts upon gene expression (Chen et al., 2000; Dennler et al., 1998; Hu et al., 2003; Verrecchia et al., 2001a).

3.1.1 Transforming growth factor-beta (TGF- β)

TGF- β is the founding member of the TGF- β superfamily comprised of over 30 multifunctional peptide cytokines in mammals including activins (Munz et al., 2001), inhibins (Mather et al., 1997), bone morphogenetic proteins (BMPs) (Kingsley, 1994), myostatin (Wagner, 2005) and TGF- β s (Branton and Kopp, 1999; Leask and Abraham, 2004). Members of the TGF- β superfamily are characterised by a cysteine 'knot' of six of these residues at the carboxy terminal (Yin et al., 1998; Sporn et al., 1986). TGF- β s are synthesised by many cell types including macrophages and fibroblasts as large

inactive precursor proteins which are modified intracellularly and secreted as a latent complex (Rifkin, 2005). Although still poorly understood several factors have been shown to promote the release of bioactive TGF- β from this latent complex including, MMPs (Mu et al., 2002), ROS (Barcellos-Hoff and Dix, 1996) and thrombospondin-1 (Murphy-Ullrich and Poczatek, 2000; Daniel et al., 2004). The resultant bioactive TGF- β regulates several hundred target genes in fibroblasts promoting a variety of cellular effects including proliferation, differentiation in addition to promoting matrix deposition and reducing matrix turnover (Chambers et al., 2003; Yang et al., 2003; Shi-wen et al., 2006b).

Of five distinct TGF-beta isoforms sharing between 64-82% protein identities, only TGF- β 1, -2 and -3 have been reported to be expressed in mammals (Massague, 1990). Encoded by separate genes, mammalian TGF- β s are differentially regulated by their promoters resulting in tissue specific expression (Kaartinen et al., 1995; Sanford et al., 1997; Kulkarni et al., 1993; Roberts et al., 1991). TGF- β s exhibit similar *in vitro* biological activities, although *in vivo* the different expression patterns of TGF- β are likely to account for the isoform specific differences in the phenotypes observed in TGF- β knockout mice (Roberts et al., 1991). TGF- β 1 null mice die from vascular and haematopoietic defects either *in utero* or soon after birth (Dickson et al., 1995; Kulkarni et al., 1993), whereas TGF- β 2 null mice die prenatally due to malformation of multiple organs (Sanford et al., 1997) and TGF- β 3 null mice die shortly after birth as a result of defects in pulmonary and palate formation (Kaartinen et al., 1995; Proetzel et al., 1995).

3.1.2 The TGF- β family receptors

The pleiotropic effects of all the TGF- β superfamily members are transmitted from membrane to nucleus through the activation of serine/threonine kinase receptors and downstream effectors, which lead to the transcriptional regulation of target genes. In fibroblasts, like other cell types, all TGF- β s signal via the sequential binding and activation of type II and type I serine-threonine receptors and the activation of downstream signalling pathways. Interestingly, BMP receptors, in addition to sequential activation of type II and recruitment of type I receptors, are present as pre-formed type II/ type I receptor complexes, independently of BMP ligand. Further these receptor complexes, pre-formed verses sequentially bound, exert differential effects on downstream signalling pathways (Nohe et al., 2002; Hassel et al., 2003). However, it remains unclear the role played of pre-formed receptor complexes in TGF- β signalling. To date

five type II and seven type I (activin receptor-like kinases; ALKs) receptors have been identified in vertebrates. Structurally similar, these TGF- β receptor sub-families are composed of three regions: a short cysteine-rich extracellular domain, a single transmembrane domain, and intracellular domains with a serine/threonine kinase region (Derynck and Zhang, 2003). All three TGF- β isoforms bind exclusively to type II receptor T β RII, and one of three type I receptors, ALK-1, ALK-2 and ALK-5. In addition two TGF- β co-receptors have been identified, betaglycan and endoglin (CD105). In general, TGF- β receptors bind more efficiently to TGF- β 1 and - β 3 than to - β 2 (Qian et al., 1996; Cheifetz et al., 1987). High affinity binding of TGF- β 2 is dependent upon betaglycan, which is, like type I and type II receptors, expressed on most cell types including fibroblasts (Lopez-Casillas et al., 1993; Stenvers et al., 2003; Attisano et al., 1993). In contrast, TGF- β 1 and - β 3 are preferentially bound by the accessory receptor endoglin, which is highly expressed on endothelial cells and weakly expressed on fibroblasts (Lebrin et al., 2004; Leask et al., 2002a; Cheifetz et al., 1992). *In vitro*, all three TGF- β isoforms stimulate fibroblast expression collagen type I (Yu et al., 2003b; Coker et al., 1997), but not *in vivo* (Kinbara et al., 2002). The different affinities and requirement of accessory receptors have been suggested to partially explain these differences (Ferguson and O'Kane, 2004; Kinbara et al., 2002). In the majority of cell types TGF- β binding activates the type I receptor ALK-5 (Agrotis et al., 1996; ten Dijke et al., 1994). However in endothelial cells, where ALK-1 is predominantly expressed, TGF- β activates signalling via ALK-1 and ALK-5 (Lebrin et al., 2004). *In vitro* these co-receptors act in both a positive and negative manner. For example, TGF- β 1 induction of CTGF is repressed by excessive expression of endoglin (Leask et al., 2002a), whereas over-expression of betaglycan enhances CTGF expression (Santander and Brandan, 2006).

3.1.3 TGF- β signalling

TGF- β s bind to a homodimeric, constitutively active serine/threonine kinase T β RII and in turn a homodimer comprised of T β RI is recruited, forming a heterotetrameric complex (Yamashita et al., 1994). The formation of this complex results in type II receptor phosphorylation of the 'GS domain' in the type I receptors which is both essential and sufficient for TGF- β signalling (Persson et al., 1998; Attisano et al., 1993). The binding of TGF- β isoforms and activation of T β RI in turn leads to the activation of downstream intracellular signalling pathways. The main downstream signalling component activated by TGF- β , and members of the TGF- β superfamily, are the Smads.

3.1.4 Smad signalling

The Smad protein family was first identified as substrates of TGF- β type I receptors in the mid 1990s (Zhang et al., 1996). Named by virtue of their homology to *Drosophila mothers against dpp* (*Mad*) and *C. elegans Sma* genes, this family is comprised of eight vertebrate Smads, Smads 1-8. Smads are sub-divided into three groups based upon function (see Figure 3.1): Group 1, the Receptor-regulated Smads (R-Smads), which interact with and are phosphorylated by activated T β RI; Group 2, the common pathway or co-operating Smads (Co-Smads) that are recruited by phosphorylated R-Smads and serve as a common partner to R-Smads; and Group 3, the inhibitory Smads (also termed Anti-Smads) which act as decoys competing with R-Smads. R-Smads and Co-Smads are characterised by highly homologous globular amino and carboxyl-terminals, named MH-1 and MH-2 respectively (Shi et al., 1998) separated by a proline rich linker region (Figure 3.1). termed the L45 loop (Feng and Derynck, 1997).

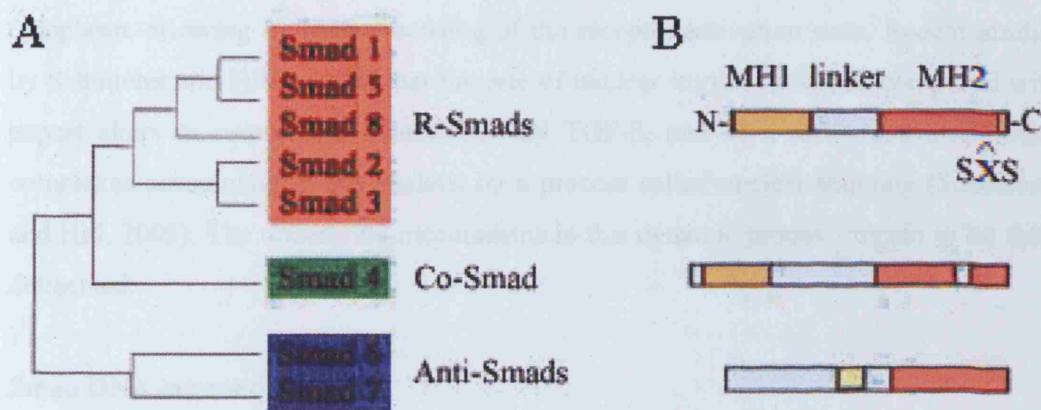


Figure 3.1
The Smad family.

A simplified dendrogram of the sequence similarity between the three Smad subfamilies (A). Structurally the receptor-regulated Smads (R-Smads) and the cooperating Smad (Co-Smad) contain a conserved MH1 (N-terminal) and MH2 (C-terminal) domains. Inhibitory Smads (Anti-Smads) contain only the MH2 domain. SXS represents the receptor phosphorylated site at the carboxyl-terminus of the R-Smads (B). Modified from Derynck *et al.* (Feng and Derynck, 2005; Derynck and Zhang, 2003).

Smads2 and 3 serve principally as substrates for TGF- β s, activins and nodal receptors T β RI (ALK5) and ActRIB. Smads 1, 5 and 8 are activated by the remaining type I receptors (ALK1, ALK2, ALK3 and ALK6) in response to BMPs, other TGF- β superfamily members, in addition to TGF- β type I receptors ALK1 and ALK2. Unstimulated Smads are predominantly anchored in the cytoplasm (Dong et al., 2000); however recent studies suggest unactivated Smads may shuttle between the nucleus and cytoplasm (Schmierer and Hill, 2005) through direct interactions with nucleoporins (Xiao et al., 2000). Upon ligand receptor interactions a cascade of signalling events occurs (Figure 3.2). All TGF- β isoforms principally activate the R-Smads, Smad2 and Smad3 via the ALK5 receptor. Smad2 and Smad3 are recruited to the receptor ligand complex by SARA (Smad Anchor for receptor activation) (Tsukazaki et al., 1998). Upon ligand binding to ALK5, the R-Smads become phosphorylated by the type I receptor, leading to their activation and dissociation from the receptor complex (Macias-Silva et al., 1996; Liu et al., 1997). The phosphorylation of R-Smads leads to a conformational change in the Smad complex forming a binding site for Smad4 (Chacko et al., 2004). The trimeric complex which then forms, consisting of two R-Smads and one Co-Smad, accumulates in the nucleus (Chacko et al., 2004; Lagna et al., 1996; Hata et al., 1997). Subsequent dephosphorylation of R-Smads causes them to return to the cytoplasm, allowing a constant sensing of the receptor activation state. Recent studies by Schmierer and Hill suggest that the rate of nuclear import of Smads compared with export alters in response to stimulation by TGF- β , and as a result activated Smad complexes accumulate in the nucleus by a process called nuclear trapping (Schmierer and Hill, 2005). The underlying mechanisms in this dynamic process remain to be fully delineated.

Smad DNA interactions

The activated Smad complex interacts with SMAD-specific DNA-binding motifs. Complexes containing Smad3 recognise, through their MH1 domain, a specific palindromic sequence (GTCTAGAC) (Zawel et al., 1998; Shi et al., 1998). Subsequent studies have identified Smad/CAGA elements in the promoter elements of many TGF- β responsive genes, including PAI-1 and COL1A2 (Dennler et al., 1998; Chen et al., 2000). In contrast to Smad3, Smad2 complexes are unable to directly bind to DNA due to a 30 amino acid insertion in the MH1 domain (Dennler et al., 1999). Accordingly Smad2 forms a complex with co-factors, such as FAST-1 and FAST-2, in order to interact with DNA (Zawel et al., 1998; Liu et al., 1999; Chen et al., 1997). The ability of

Smads to modulate transcription in response to ligand also results from a functional cooperation with ubiquitous transcriptional coactivators or corepressors. For instance, DNA binding by Smad2 and Smad3 is promoted through interactions with p300 (Nishihara et al., 1998). Subsequently many such co-activators have been identified, including the transcription factor SP1 (Feng et al., 2000) and members of the AP-1 family (Wong et al., 1999). The recruitment of corepressors can also inhibit these interactions; for example TG-interacting factor (TGIF) interferes with the assembly of Smad2 and the coactivator p300 (Pessah et al., 2001).

Down regulation of TGF- β Signalling

Termination of the Smad signalling pathway is regulated by a negative auto-feedback loop of the inhibitory Smads, Smad6 and Smad7. These 'antagonists' of TGF- β signalling lack the C-terminal domain SXS phosphorylation site, preventing Smad-receptor interaction and subsequent down-stream phosphorylation events (Figure 3.1). Induced by the BMPs, Smad6 is preferentially involved in the inhibition of BMP (Smads 1, 5 and 8) signalling (Hata et al., 1998). In addition Smad6 also inhibits Smad2 but not Smad3 (Imamura et al., 1997). Smad7, potently induced by TGF- β in a Smad3 dependent manner (Nagarajan et al., 1999), inhibits the TGF- β activated Smad2 and Smad3 pathways (Nakao et al., 1997).

In addition to I-Smads, TGF- β signalling is down regulated through receptor and R-Smad degradation by Smad ubiquitin regulatory factors (Smurfs) and inhibition of the R-Smad complex by Ski and SnoN. Interaction of Smurfs with the R-Smads through a specific PY motif targets their degradation to maintain a low basal level in the absence of signalling. In an alternate process that is signalling dependent, the Smurfs can target activated type I receptors and enhance receptor turnover (Arora and Warrior, 2001; Asano et al., 2004b). The protooncogenes Ski and SnoN directly repress TGF- β through interaction with a C-terminal region of the MH2 domain in Smad3 (Mizuide et al., 2003). Collectively these mechanisms lead to the cessation of TGF- β signalling.

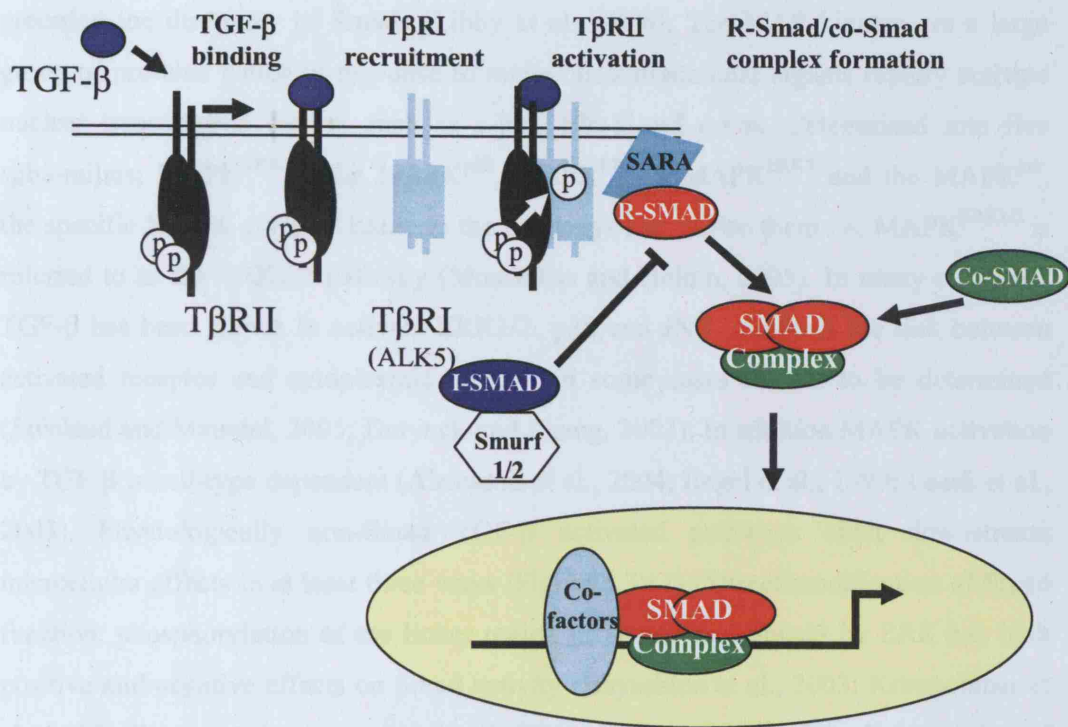


Figure 3.2

Schematic diagram of the TGF-β Smad signalling pathway.

TGF-β activates the Smad to elicit numerous biological processes. TGF-β binding to their cognate receptors results in the recruitment of R-Smads- Smad 2 or Smad3 in complex with SARA. Phosphorylation by the receptor complex leads to the dissociation of R-Smads from SARA and the recruitment of the Co-Smad, Smad 4. The resulting trimeric 'Smad complex' of Smad4 and two homomeric R-Smads translocates to the nucleus. Transcriptional activity of the Smad complex is modulated by the binding of co-factors. The inhibitory I-Smad, Smad7 and Smurfs in turn block the interaction of R-Smads with the TGF-β receptor. Modified from Massague *et al.* (Massague, 1998; Kretzschmar *et al.*, 1999).

3.1.5 Non-Smad signalling pathways

Although Smads have firmly established themselves at the heart of TGF- β signalling, other signalling cascades are activated by TGF- β , including the mitogen activated protein kinase (MAPK) pathways. The identification of non-Smad signalling pathways preceded the discovery of Smads (Libby et al., 1986). The MAP kinases are a large group of proteins which in response to numerous extracellular signals rapidly activate nuclear transcription factors such as c-jun, Elk-1 and c-Fos. Categorised into five subfamilies; MAPK^{ERK1/2}, the MAPK^{p38}, MAPK^{ERK3/4}, MAPK^{ERK5} and the MAPK^{jnk}, the specific MAPK gives its name to the pathways that utilise them i.e. MAPK^{ERK1/2} is referred to as the ERK1/2 pathway (Moustakas and Heldin, 2005). In many cell types TGF- β has been shown to activate ERK1/2, p38 and JNK, although the link between activated receptor and cytoplasmic effector in some cases remain to be determined (Javelaud and Mauviel, 2005; Derynck and Zhang, 2003). In addition MAPK activation by TGF- β is cell-type dependent (Abecassis et al., 2004; Engel et al., 1999; Leask et al., 2003). Physiologically non-Smad TGF- β activated pathways elicit downstream intracellular effects in at least three ways (Figure 3.3): (1) Direct modification of Smad function; phosphorylation of the linker region in Smad2 and Smad3 by ERK has both positive and negative effects on Smad activity (Hayashida et al., 2003; Kretzschmar et al., 1999). For example in mesangial cells ERK phosphorylation of the linker region of Smad3 leads to the enhanced activation of COL1A2 (Hayashida et al., 2003), whereas the p38 substrate MSK1 kinase promotes the association of Smad3 with the co-activator p300 (Abecassis et al., 2004). (2) Functional modulation by Smads; for example JunB is expressed at low levels in fibroblasts but is transcriptionally induced by TGF- β via Smads and in turn promotes TGF- β activation of COL1 α 2 and PAI-1 (Chang and Goldberg, 1995). (3) Actions independent of Smads. For example several TGF- β responsive extracellular matrix genes and matrix regulatory enzymes such as Urokinase-type plasminogen activator receptor and fibronectin genes are regulated by MAPK pathways independent of the Smad signalling pathway (Yue et al., 2004; Hocevar et al., 1999).

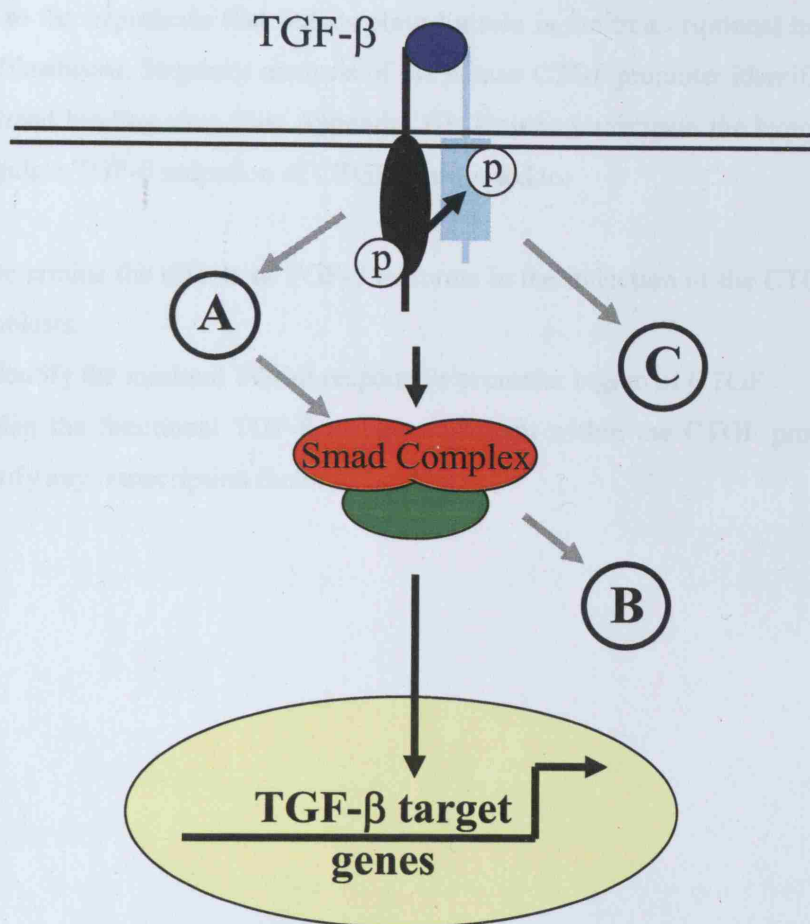


Figure 3.3

Schematic of the direct and modifying effects of the non-Smad signalling pathways.

TGF- β ligand-receptor complex activates the Smad signalling pathway and target genes in the nucleus (Black arrows). Non-Smad signalling mechanisms are shown in grey arrows. The TGF- β -receptor complex directly activates protein A, which modulates the activity of the Smad pathway (A); the activated Smad complex activates protein B, which then transmits further signals into the nucleus (B); The TGF- β /receptor complex directly activates protein C, which signals independently of Smads (C). Modified from Moustakas *et al.* (Moustakas and Heldin, 2005).

3.2 Aims

The identification of Smads as the major mechanism of TGF- β activation of target genes led to the hypothesis that Smads played a role in the transcriptional induction of CTGF in fibroblasts. Sequence analysis of the human CTGF promoter identified several putative Smad binding sites (See Appendix II). Thus to investigate the hypothesis that Smads regulate TGF- β induction of CTGF, I proposed to:

- 1- Determine the effects of TGF- β isoforms in the induction of the CTGF gene in fibroblasts.
- 2- Identify the minimal TGF- β responsive promoter region of CTGF.
- 3- Map the functional TGF- β response element within the CTGF promoter and identify any transcription factor(s) binding.

3.3 Materials and Methods

3.3.1 Generation of CTGF promoter construct

3.3.1.1 Culture and purification of Pac clone, RP3-417F7 (AL133346)

The DNA PAC clone RP3-417F7 (kind gift Dr Andrew Mungall, Sanger Centre) was transformed into the *E.coli* strain DH5 α as described in Section 2.3.3. DNA was isolated using the Qiagen large construct kit (Qiagen) as per the manufacturer's instructions. Briefly, an overnight culture of the *E.coli* strain DH5 α containing the PAC clone, RP3-417F7, was inoculated into 500 ml of LB and agitated overnight. DNA was prepared as described in Section 2.3.4.2 taking care to avoid vortexing to prevent DNA shearing. Upon binding of the PAC DNA to the Qiagen-tip 500, ATP-dependent exonuclease was applied to digest any contaminating genomic and nicked PAC clone DNA, leaving only supercoiled PAC DNA remaining. The DNA pellet was resuspended in an appropriate volume of TE buffer and the concentration determined by UV spectrophotometer (Hitachi U-2001).

3.3.1.2 DNA Ligation

The plasmids pGL3 (Promega), CTGF800 and the Pac clone, RP3-417F7 were digested with the appropriate restriction enzymes as indicated in Figure 3.5 and fragments size separated by agarose gel electrophoresis as described in Section 2.3.6. Fragments were isolated and concentrations determined by UV spectrophotometer. Ligations containing equal molar ratios of DNA fragments from RP3-417F7 (SpeI/ NotI digested), pCTGF800 (NotI/XhoI digested) and linearized pGL3 (basic) (NheI/XhoI digested) were carried out in 20 μ l 10 mM Tris-Cl pH 7.4, 10 mM Mg²⁺, 1 mM ATP, 1 mM DTT, using 0.2 - 1.0 U T4DNA ligase (Promega) and 10 ng DNA, at 16°C for 16 h.

3.3.1.3 Generation of deletion and point mutation constructs

CTGF promoter reporter deletion and point mutation constructs were generated in collaboration with Dr Andrew Leask. Briefly, a CTGF promoter deletion series was generated using the CTGF luciferase plasmid as a template (Grotendorst et al., 1996). Promoter fragments of various lengths were amplified by polymerase chain reaction (PCR), vent polymerase (New England Biolabs) and oligonucleotides (Table 3.1) designed to amplify specific regions of the CTGF promoter (See Appendix II). Oligonucleotide primers -805, -244, -166 and -86 annealed to the CTGF promoter as indicated (See Appendix II), 5' to the transcriptional start site of CTGF and contained

the KpnI restriction enzyme site (Table 3.1) and a 3' anti-sense oligonucleotide, +17, containing an XhoI site (Table 3.1), annealed to the CTGF promoter 3' to the sequence AACT⁺¹CAC (See Appendix II). A PCR reaction mixture containing- 100ng of the CTGF/luc vector as template; 250ng ml⁻¹ of each oligonucleotide primer 48 µl of master mix (5 µl 10X Buffer (Cambio, UK), 10 mM dNTPs, 1 unit of vent polymerase (New England Biolabs) and 2.5mM MgCl₂)- was over laid with oil and placed in an automated thermal cycler PTC-100 (MJ research, Inc). After being initially denatured for 5 mins at 94°C, CTGF promoter fragments were amplified for 35 cycles (94°C- 10 s, 55°C- 40s, 72°C- 2mins). PCR products were digested with KpnI and XhoI (Chapter 2; Section 2.3.5), resolved by agarose gel electrophoresis (Chapter 2; Section 2.3.6), excised and gel purified (Chapter 2; Section 2.3.7). Isolated CTGF promoter fragments were ligated to KpnI/ XhoI digested SEAP basic vector (Invitrogen). Point mutations in the CTGF promoter were generated using Quik Change (Stratagene) system by Dr Andrew Leask. Point mutations in the CTGF promoter were introduced using mutagenic primers (MWG Biotech), as indicated in Appendix II, The resultant constructs were fully sequenced in both directions to confirm mutagenesis before use.

Construct generated		PCR Oligonucleotide
CTGF800	-805	5' GAAGGTACC TGATAGAACAATGGATC 3' S
	+17	5' CAACTCGAG GAAGAGTTGTTGTGTGAG 3' AS
CTGF244	-244	5' GAAGGTACC GCTGGAAATACTGCGCT 3' S
	+17	5' CAACTCGAG GAAGAGTTGTTGTGTGAG 3' AS
CTGF166	-166	5' GAAGGTACC GAATGCTGAGTGTC AAGG 3' S
	+17	5' CAACTCGAG GAAGAGTTGTTGTGTGAG 3' AS
CTGF86	-86	5' GAAGGTACC CTGTTTGTGTAGGACTCC 3' S
	+17	5' CAACTCGAG GAAGAGTTGTTGTGTGAG 3' AS

Table 3.1

Generation of CTGF deletion constructs.

Using the CTGF luciferase plasmid as template, promoter fragments of various lengths were amplified using PCR using primers indicated (Also see Appendix II). S-denotes sense and AS-denotes anti-sense oligonucleotide. Sense (S) oligonucleotides contained a Kpn I (GGTACC) whereas the anti-sense (AS) oligonucleotide contained the Xho I restriction enzyme site (CTCGAG) as indicated.

3.3.2 Cell culture

3.3.2.1 Cell lines

Five adherent cell lines were used in these studies; HFF, murine NIH/3T3 and primary human dermal fibroblasts are described in Section 2.2. Murine foetal and newborn dermal Smad 3 null and wild type counterparts were a generous gift of Anita Roberts (Yang et al., 1999)

3.3.2.2 Routine propagation of cell lines

Foetal Smad3 null and wild type cells were maintained at 37°C in 5% CO₂, in Dulbecco's modified Eagle medium (DMEM; GIBCO) supplemented with 20% foetal calf serum (FCS) and 2 mM L-glutamine. All other cell types were maintained as described in Section 2.2. Cells were passaged when confluent by trypsinization of the cell monolayer (0.125% trypsin in PBS, 0.5 mM EDTA), and seeded into cell culture flasks at a density of 1:3 depending on the requirement of experiments.

3.3.2.3 Optimisation of transfection efficiencies- β -Galactosidase staining

Transfection efficiencies of HFF, NIH/3T3, and primary dermal fibroblasts cell lines were determined using FuGENE 6 (Roche) and Lipofectamine Plus (Invitrogen) and performed as described in Section 2.2.4. The efficiency of the transient transfections was optimized using the LacZ expression vector, pSV- β -Galactosidase. Fibroblast cell lines were grown to 80% confluence in 12 well plates and then transfected with 500 ng of pSV- β -Galactosidase with a range of FuGENE 6 and Lipofectamine (Table 3.1). Where Lipofectamine was used for transfection, a fixed amount of Plus reagent (4 μ l) was used. After 24 h cell monolayers were washed in PBS and fixed (0.2% Glutaraldehyde, 0.1M Na Pi Buffer, 5mM EGTA (pH 8.0), 2mM MgCl₂, 2% Formalin) with gentle agitation. After 10 min the monolayer was washed twice in rinse solution (0.1M NaPi Buffer, 2mM MgCl₂, 0.1% Na deoxycholate, 0.2% NP40). Cell monolayers were then stained for β -Galactosidase in LacZ staining solution (1 mg/ml 5-bromo-4-chloro-3-indolyo- β -D-galactoside solution (X-gal) containing 5 mM potassium ferrocyanide and 5 mM potassium ferricyanide) and stained at room temperature in the dark for 4-8 h. The monolayer was washed in PBS and transfection efficiency as measured by the percentage of LacZ/Blue staining cells per field of view calculated. All three fibroblast cell lines exhibited the highest percentage transfection efficiency using 2 μ l of Lipofectamine and 4 μ l PLUS reagent, with primary dermal cell lines exhibiting the lowest transfection efficiency (Table 3.2). All subsequent transfections were

performed using Lipofectamine Plus reagent and transfections performed in either 6 or 12 well tissue culture plates. Where 6 well plates were used, transfection reagents and DNA were scaled accordingly.

Cell Type	Transfection reagent				
	Lipofectamine (μ l)			FuGENE 6 (μ l)	
	1 μ l	2 μ l	6 μ l	2 μ l	4 μ l
NIH/3T3	25-60%	35-70%	45-60%	40-58%	45-65%
HFF	10-16%	15-25%	10-17%	4-11%	7-15%
Dermal	2-8%	8-11%	4-10%	6-10%	8-10%

Table 3.2

Optimal transfection efficiencies of fibroblast.

Efficiencies of transfection of primary and immortal fibroblast cell lines using Lipofectamine Plus (4 μ l Plus reagent used) and FuGENE 6 as percentage of LacZ/Blue staining cells per field of view.

3.3.3 Statistical analysis

All results are expressed as means \pm SEM unless otherwise stated. Student's unpaired-test was used for statistical analyses. P-values less than 0.05 were considered statistically significant.

3.4 Results

3.4.1 Induction of endogenous CTGF by TGF- β

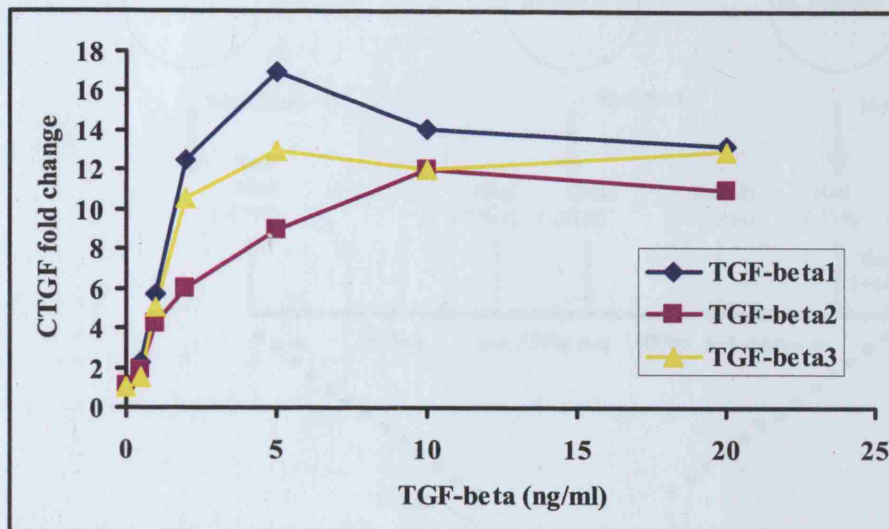
Quantitative real-time PCR detected low levels of CTGF mRNA expression in all three serum-starved fibroblast cell lines. However all three cell types demonstrated a robust and rapid time dependent induction in response to TGF- β 1. These studies confirmed previous published observations (Shi-wen et al., 2000; Grotendorst et al., 1996). Maximal induction of CTGF mRNA by TGF- β was observed at 6 h and returned to basal levels after 24h (Figure 3.4). All three TGF- β isoforms, - β 1, - β 2 and - β 3, elicited similar inductive responses yielding greater than 9-fold induction of CTGF transcription compared to baseline expression in HFF cells. Of the three isoforms, TGF- β 1 induced the greatest expression (17 fold). Both TGF- β 1 and - β 3 induced maximal responses at concentrations of 5ng ml⁻¹. The highest CTGF induction was observed at 10 ng ml⁻¹ in response to TGF- β 2 (Figure 3.4).

3.4.2 Generation of the CTGF promoter constructs

Previously, Grotendorst *et. al.* had identified a TGF- β 1 response element in the proximal region of the CTGF promoter. Promoter scanning showed this region to share no significant homology to known TGF- β response elements or the subsequently identified Smad3 consensus DNA binding site. The genomic PAC clone RP3-417F7 (Accession number AL354866), containing 32 kb of human chromosome 6q22.31-23.3, was analysed for unique restriction enzyme sites spanning the CTGF 5' UTR region using Webcutter 2.0 (<http://rna.lundberg.gu.se/cutter2/>). Two unique restriction enzyme sites spanning a 4.3 kb region of 5' UTR of CTGF were identified, SpeI at position -4541, Not I at position -170, upstream of the transcription start site (Grotendorst et al., 1996) to +54 (Figure 3.5A). The PAC clone was digested with restriction enzymes and the 4.3 kb SpeI/ NotI DNA fragment isolated by agarose gel electrophoresis. The previously reported CTGF promoter construct, pCTGF800, was digested with NotI and XhoI corresponding to the first ~200 bp of the CTGF proximal promoter region, containing the TATAA box (Grotendorst et al., 1996) and agarose gel purified. These CTGF promoter fragments were ligated into a linearised Nhe I/Xho I digested reporter vector, pGL3 (Promega), in equal molar ratios. The NheI (pGL3) and SpeI sites (5' end of CTGF fragment) were destroyed as a consequence of the cloning. A schematic of the cloning strategy is shown in Figure 3.5. Three clones were isolated, and mapped by restriction digestion (Figure 3.5), and compared to the predicted restriction map

generated by the program, Webcutter 2.0. The confirmed construct was named pCTGF4.5. The validity and integrity of one clone, clone #4, was further confirmed by direct DNA sequencing (Appendix II).

A



B

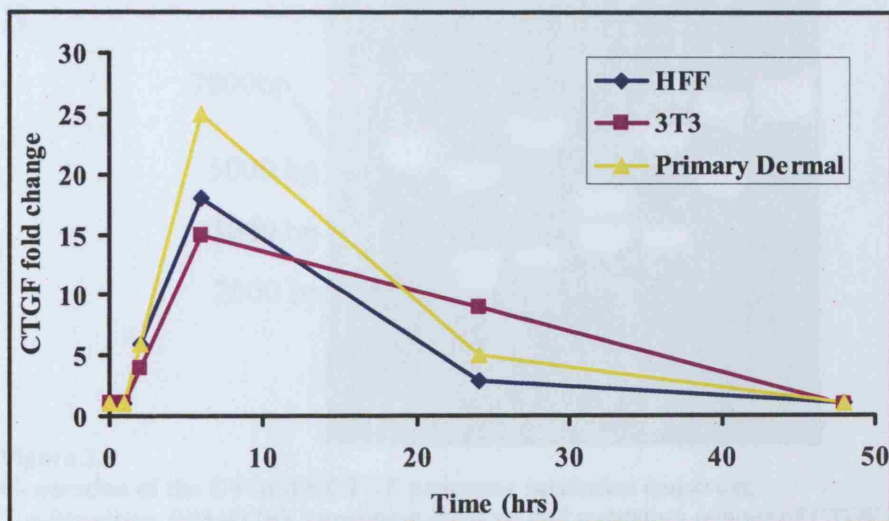


Figure 3.4

Time and dose dependent induction of CTGF by TGF- β .

Fibroblasts were grown to 80% confluence and serum starved for 24 h. Cells were then incubated for 6 h in 0, 0.5, 1, 2, 5, 10 and 20 ng ml⁻¹ of rTGF- β 1, - β 2 and - β 3. RNA was isolated and real time PCR for CTGF performed. Values were normalized against 28S rRNA expression and fold change plotted (A). HFF, NIH/3T3 and primary dermal fibroblasts were incubated with TGF- β 1 (2 ng ml⁻¹) and CTGF mRNA expression assessed by real time PCR at 0, 2, 4, 6, 24, and 48 h and fold change plotted (B). Line graph is representative of two independent experiments.

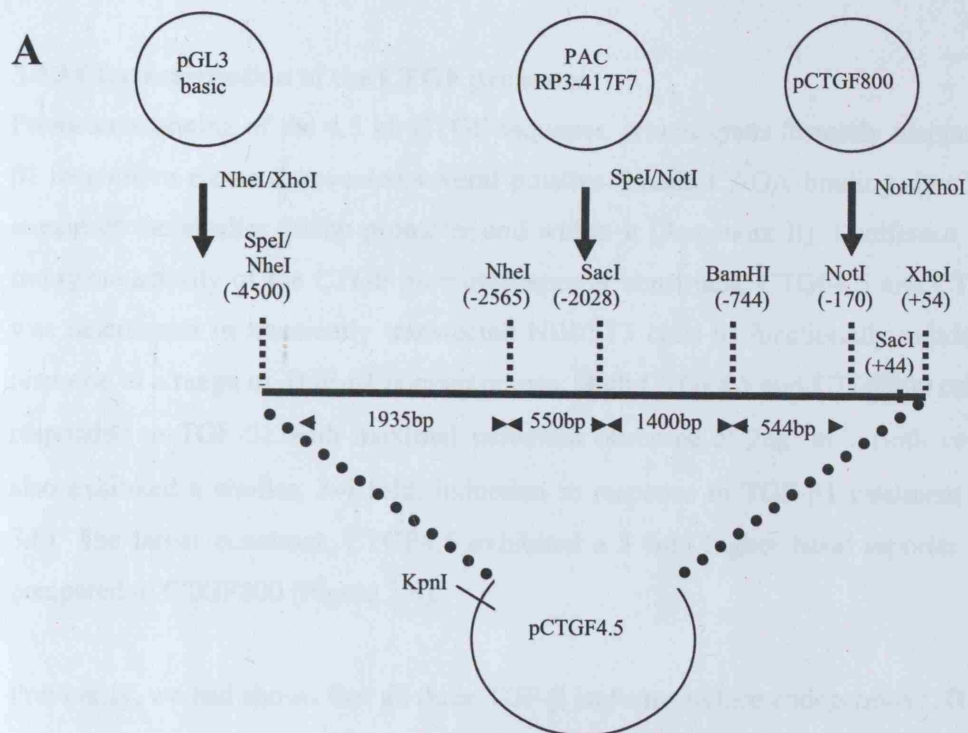


Figure 3.5

Generation of the CTGF4.5 CTGF promoter luciferase construct.

The Pac clone, RP3-417F7, containing the gene and regulatory regions of CTGF was restriction digested with SpeI and NotI and a 4.3kb fragment isolated. A 170 bp Not I/XhoI region of the proximal promoter of CTGF was isolated from the vector pCTGF800 containing 800 bp of the promoter region defined by Grotendorst *et al* (Grotendorst et al., 1996), and ligated to linearized NheI/ XhoI digested pGL3 and 170 bp of the proximal promoter from pCTGF800 was similarly ligated to pGL3. A schematic of the cloning strategy is shown (A). Clones were isolated and digested to confirm the integrity: **lane 1** Marker; **lane 2** uncut; **lane 3** SacI/KpnI; **lane 4** SacI; **lane 5** BamHI/KpnI, **lane 6** BamHI; **lane 7** KpnI/NotI; **lane 8** KpnI; A representative series of restriction digests are shown (B). All restriction enzymes were used at 10units μg^{-1} .

3.4.3 Characterisation of the CTGF promoter

Promoter scanning of the 4.5 kb CTGF sequence, which spans formerly mapped TGF- β 1 responsive element, revealed several putative Smad3 CAGA binding sites both up stream of the smaller 800bp promoter and within it (Appendix II). Luciferase reporter transgene activity of the CTGF promoter/reporter constructs, CTGF4.5 and CTGF800, was determined in transiently transfected NIH/3T3 cells to functionally validate their response to a range of TGF- β 1 concentrations. Both CTGF4.5 and CTGF800 constructs responded to TGF- β 1 with maximal induction observed at 2ng ml⁻¹. Both constructs also exhibited a similar, 3-4 fold, induction in response to TGF- β 1 treatment (Figure 3.6). The larger construct, CTGF4.5 exhibited a 3 fold higher basal reporter activity compared to CTGF800 (Figure 3.6).

Previously, we had shown that all three TGF- β isoforms induce endogenous CTGF gene activity (Figure 3.4). In NIH/3T3 and HFF cells transiently transfected with the CTGF promoter/reporter constructs CTGF4.5 or CTGF800, all three TGF- β isoforms induced a 2-4 fold increase in luciferase activity. Whereas TGF- β 1 induced a 3-4 fold induction compared to basal expression, TGF- β 2 and TGF- β 3 induced 2-3 fold increase in reporter gene activity in both cell types at the same concentrations (2ng ml⁻¹) (Figures 3.7).

Past reports have shown CTGF to be transcriptionally induced by factors other than TGF- β including ET-1 and thrombin (Xu et al., 2004; Chambers et al., 2000). In NIH/3T3 cells transfected with either the CTGF4.5 or the CTGF800 promoter/reporter constructs, treatment with ET-1 (100nM) or TGF- β 1 (2ng/ml) produced a 2 and 3 fold induction in luciferase reporter gene activity respectively. However, treatment with thrombin (1unit ml⁻¹) induced a 3 fold induction in reporter gene activity with the CTGF4.5 promoter/reporter construct whereas the smaller CTGF800 promoter/reporter construct was insensitive to stimulation by thrombin (Figure 3.8). An identical response to these ligands was observed in HFF transfected with either CTGF promoter construct (Figure 3.8). No significant ($P>0.05$) response was noted from reporter gene pGL3 alone in response to TGF- β , ET-1 or thrombin treatment in either cell type (Figures 3.8). Thus, although the larger promoter/reporter construct (CTGF4.5) was able to respond to TGF- β (β 1 β 2, β 3), thrombin and ET-1 the smaller construct (CTGF800) was unresponsive to thrombin suggesting that ET-1 and TGF- β but not thrombin

response elements lie within the first 800 bp upstream of the TATA box in the CTGF proximal promoter.

3.4.4 Mapping of the TGF- β response element in the CTGF promoter

To identify the cis-sequences necessary for the CTGF promoter to respond to TGF- β 1, NIH/3T3 and HFF cells were transfected with a series of CTGF promoter/SEAP reporter deletion constructs (Figure 3.9) in the presence and absence of TGF- β . The level of transgene activity of the CTGF promoter reporter constructs, as measured by secreted alkaline phosphatase (SEAP), was approximately 10 fold less in HFF compared NIH/3T3 cells (Figure 3.10). Reporter gene activities in NIH/3T3 and HFF cells transiently transfected with CTGF promoter/reporter constructs containing either 800 bp or the first 244 bp of the proximal promoter were significantly ($P < 0.05$) elevated in response to 2ng ml⁻¹ TGF- β 1 (Figure 3.10). Interestingly however, no induction of CTGF promoter/reporter activity by TGF- β 1 was observed in the smaller -166bp or -86bp constructs (containing the first 166 bp and encompassing the previously characterised TGF- β RE element and 86 bp of the proximal promoter respectively) after 24 hours. Therefore the TGF- β 1 response element for NIH/3T3 and HFF cells lies between -166 and -244 bp upstream of the TATA box in the CTGF promoter. Basal activity of the background vector, pBasic-SEAP (control), was low and showed no significant response in either cell line to TGF- β 1 treatment (Figure 3.10).

A

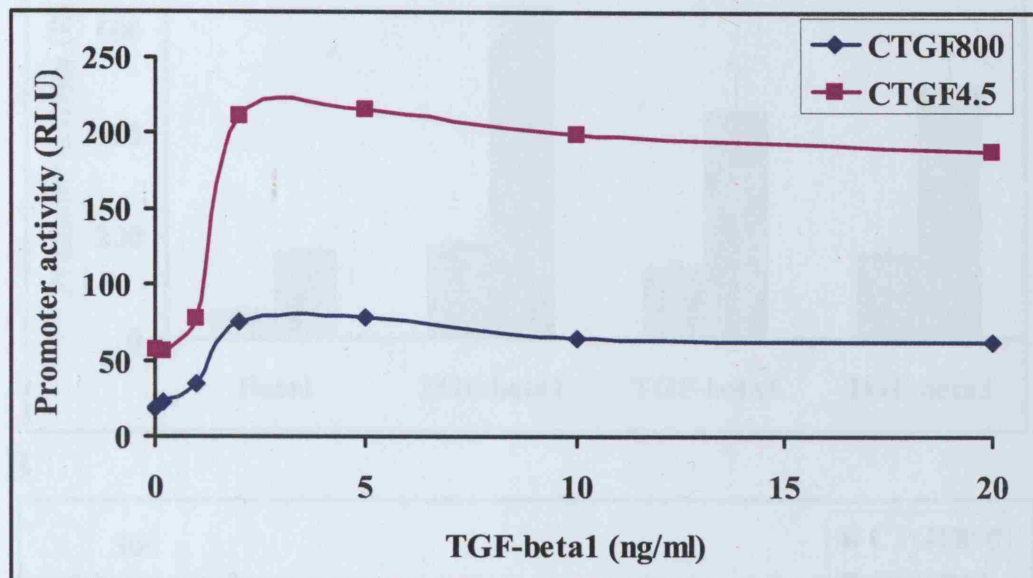
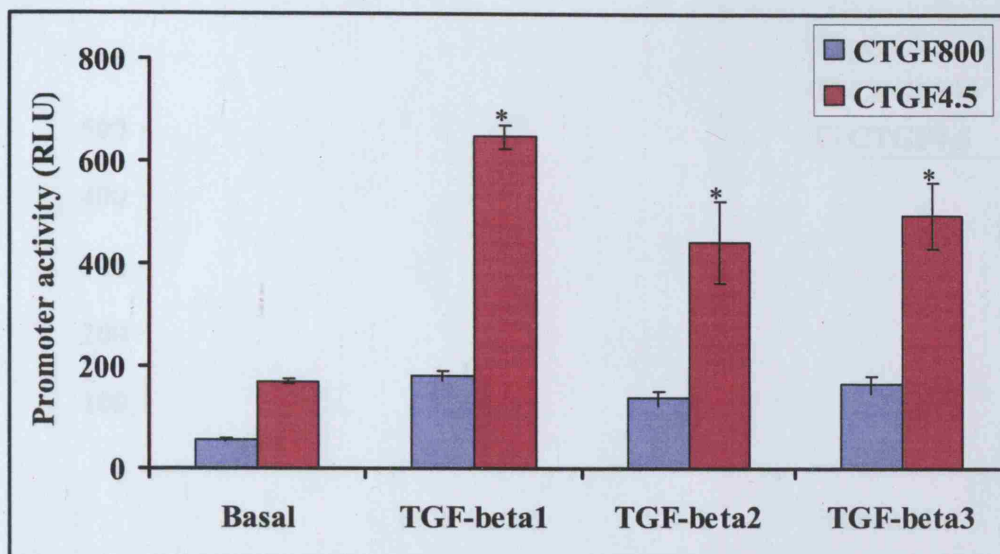
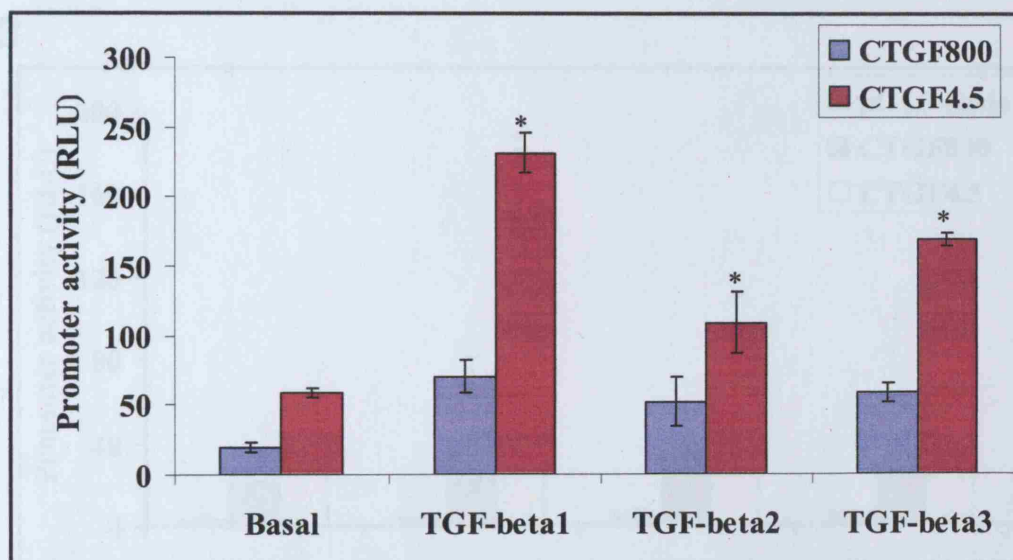


Figure 3.6

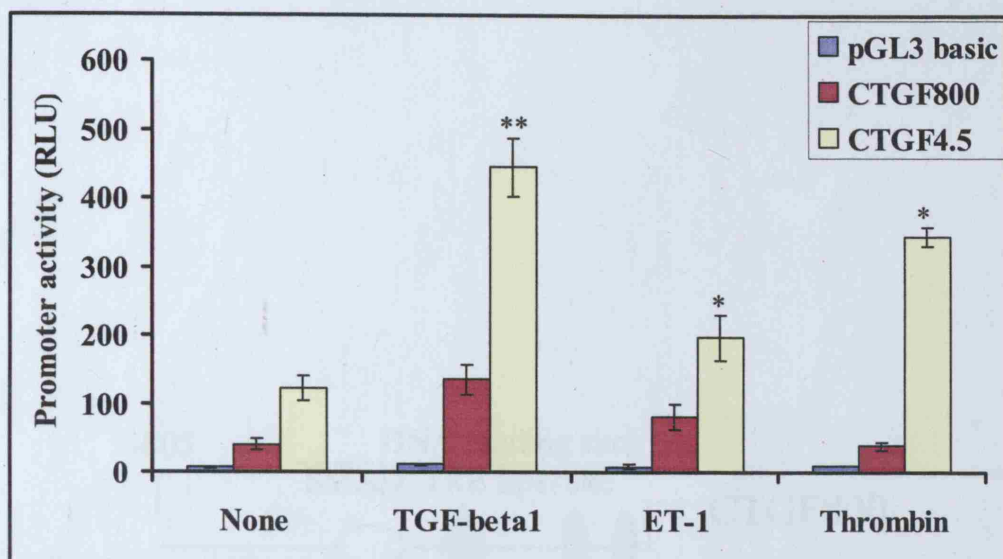
Functional characterisation of the CTGF promoter.

NIH/3T3 transiently transfected with either CTGF4.5 (Pink line) or CTGF800 (Blue line) were stimulated with 0, 0.5, 1, 2, 5, 10 and 20 ng ml⁻¹ TGF-β1 and luciferase promoter activity assessed after 24 h (A). Luciferase activity, relative light units (RLU), were normalised to β-galactosidase activity from a co-transfected CMV-β-galactosidase plasmid and mean (n=3) values plotted

A**B****Figure 3.7****Effects of TGF- β isoforms on CTGF promoter activity.**

NIH/3T3 (A) and HFF (B) were grown to 80% confluence and transiently transfected with either CTGF4.5 or CTGF800 promoter reporter construct and luciferase activity assessed in the presence of rTGF- β 1, - β 2 and - β 3 (2ng ml⁻¹) after 24 h. Luciferase activity RLU, were normalised to β -galactosidase activity from a co-transfected CMV- β -galactosidase plasmid (mean \pm SD, n=5); *p<0.05, Students unpaired t-test comparing stimulated to basal.

A



B

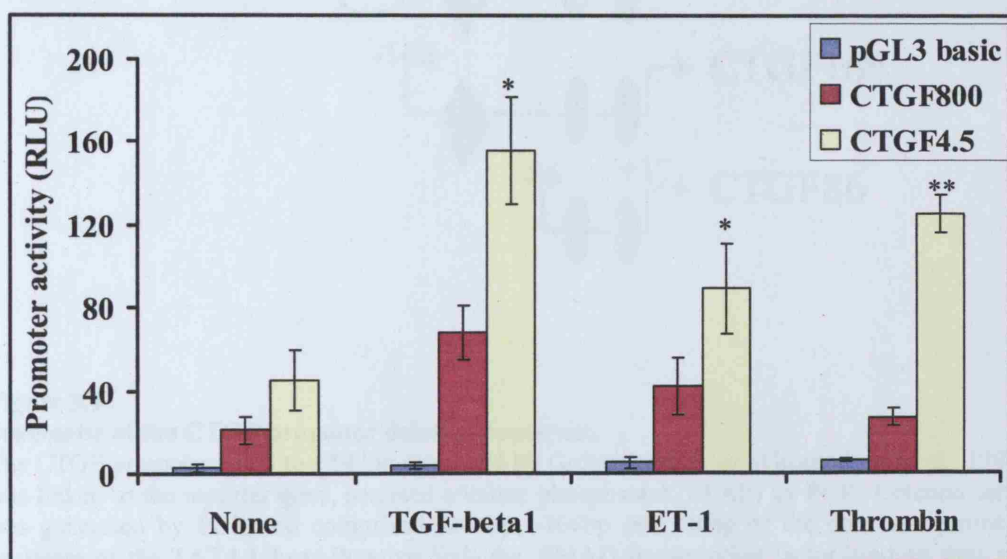


Figure 3.8

Effects of ET-1 and thrombin on CTGF promoter activity.

NIH/3T3 (A) and HFF (B) were grown to 80% confluence and transfected with CTGF4.5, CTGF800 promoter reporter constructs or the cloning vector, pGL3 basic. Luciferase activity assessed in the presence of TGF- β 1 (2ng ml⁻¹), ET-1 (100nM) or thrombin (1unit ml⁻¹) was assessed after 24 hours. Luciferase activity RLU, were normalised to β -galactosidase activity from a co-transfected CMV- β -galactosidase plasmid (mean \pm SD, n=5); *p<0.05, **p<0.02, Students unpaired t-test.

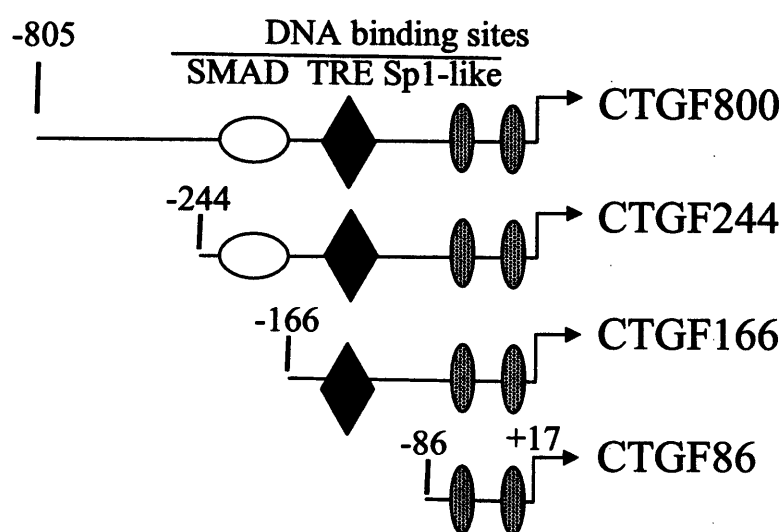
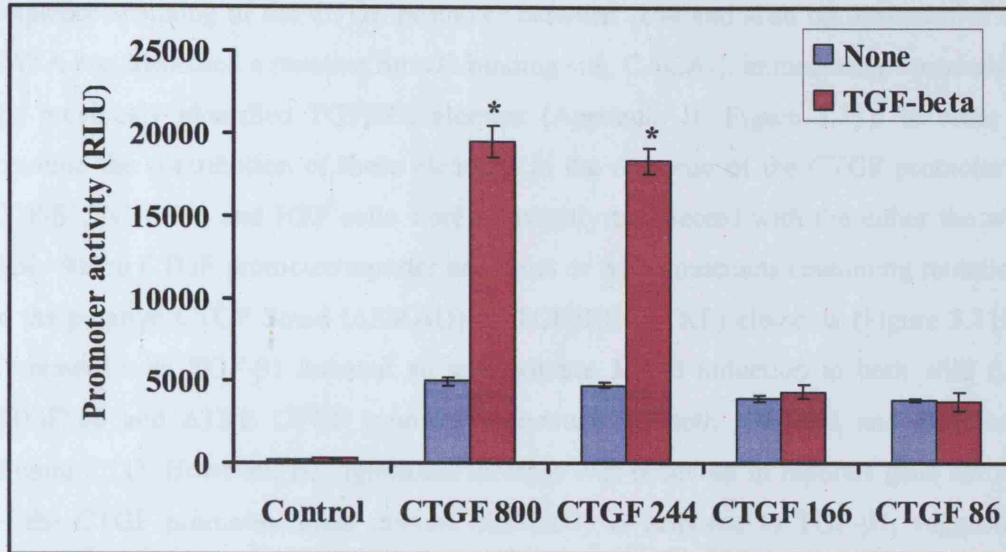


Figure 3.9

Schematic of the CTGF promoter deletion construct.

The CTGF promoter, -805 to +54, as described by Grotendorst *et al.* (Grotendorst et al., 1996), was linked to the reporter gene, secreted alkaline phosphatase (SEAP) by PCR. Deletion series was generated by PCR and comprised -244bp, -166bp and -86bp of the proximal promoter upstream of the TATAA box. Putative Sp1-like, SMAD transcription factor binding sites and the formerly reported TGF- β RE, TRE site are indicated (see Appendix II for sequence).

A



B

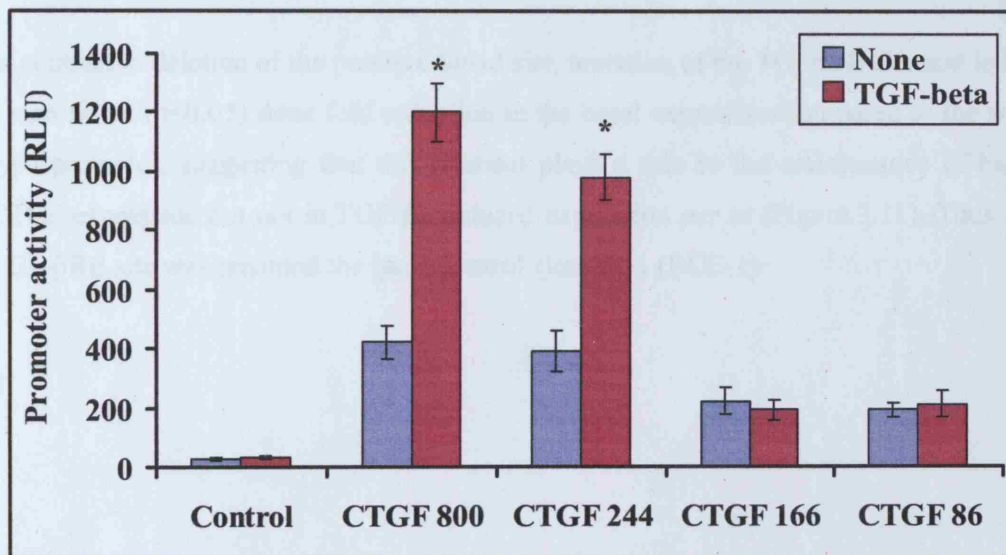


Figure 3.10

Mapping of the TGF- β response element in the CTGF promoter.

NIH/3T3 (A) and HFF (B) were grown to 80% confluence and transfected with CTGF promoter deletion constructs. SEAP promoter activity was assessed as described in Section 2.2.4.2. Relative light units (RLU) based on SEAP expression (25 μ l of conditioned medium) normalized to β -galactosidase activity from a co-transfected CMV- β -galactosidase plasmid are shown (mean \pm SEM, n=6); *p<0.05, Students unpaired t-test.

3.4.5 Deletion of the putative Smad site abolishes induction of CTGF by TGF- β 1

Sequence scanning of the CTGF promoter between -244 and -166 bp upstream of the TATA box identified a putative Smad3 binding site, CAGAC, immediately upstream of the previously identified TGF β RE element (Appendix II; Figure 3.11). In order to examine the contribution of these elements in the response of the CTGF promoter to TGF- β 1, NIH/3T3 and HFF cells were transiently transfected with either the wild type -805bp CTGF promoter/reporter construct or with constructs containing mutations in the putative CTGF Smad (Δ SMAD) or TGF β RE (Δ TRE) elements (Figure 3.11C). Treatment with TGF- β 1 induced an approximate 3 fold induction in both wild type CTGF800 and Δ TRE CTGF promoter constructs in both NIH/3T3 and HFF cells (Figure 3.11). However, no significant increase was observed in reporter gene activity of the CTGF promoter Smad mutant (Δ SMAD) in response to TGF- β 1, suggesting TGF- β 1 induction is mediated via this putative Smad transcription factor binding site (Figures 3.11).

In contrast to deletion of the putative Smad site, mutation of the TGF β RE element led to a significant ($p < 0.05$) three fold reduction in the basal expression compared to the wild type promoter, suggesting that this element plays a role in the maintenance of basal CTGF expression but not in TGF- β 1 induced expression *per se* (Figure 3.11). Thus the TGF- β RE site was renamed the basal control element-1 (BCE-1).

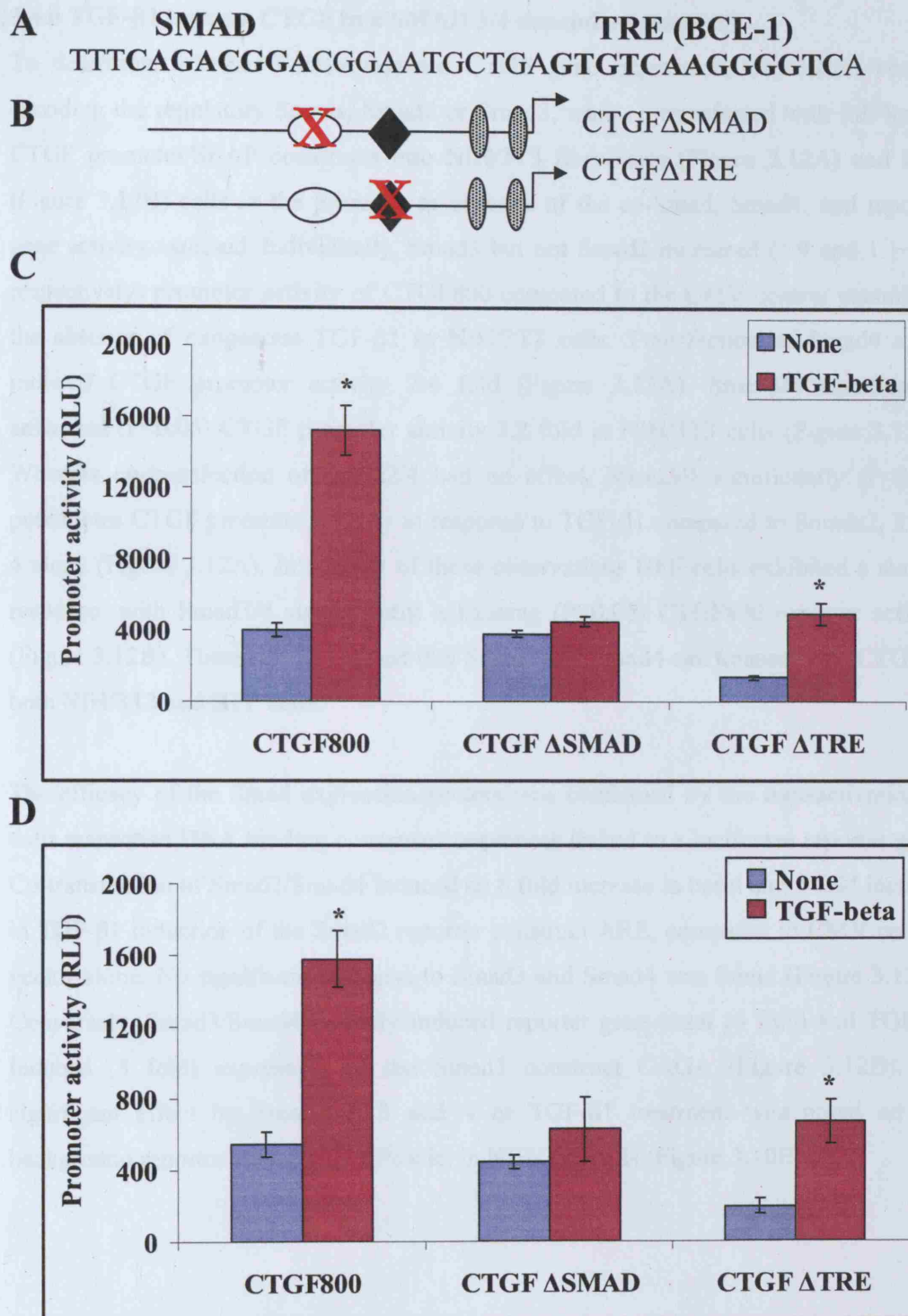


Figure 3.11

Mutation of the putative SMAD element abolishes TGF- β 1 induction of CTGF.

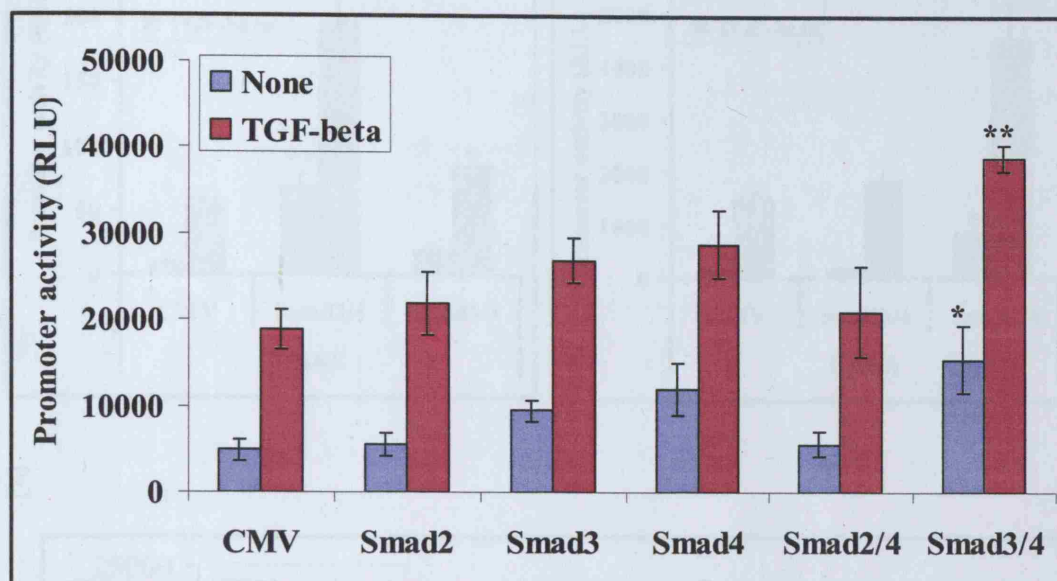
Sequence inspection of the CTGF promoter identified a putative Smad binding site upstream of the TGF β RE (TRE) element (A). Point mutations were generated in the context of the wild type -804 bp to +17bp CTGF promoter (B). NIH/3T3 (C) and HFF (D) fibroblasts were transiently transfected with CTGF800, Δ SMAD or Δ TRE (Δ BCE-1) in the presence and absence of TGF- β 1 (2ng ml⁻¹) and CTGF promoter activity, determined in RLU based on SEAP expression (25 μ l of conditioned medium), was normalized to β -galactosidase activity from a co-transfected CMV- β -galactosidase plasmid (mean \pm SEM, n=3); *p<0.05, Students unpaired t-test (C).

3.4.6 TGF- β 1 induces CTGF in a SMAD 3/4 dependent manner

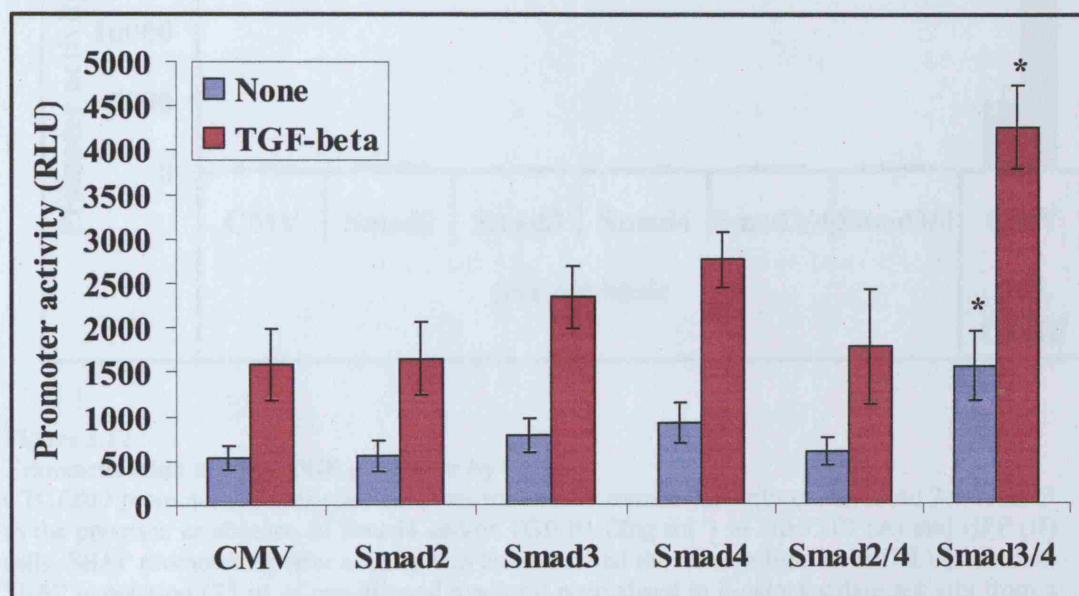
To determine whether Smads regulate CTGF gene expression, expression vectors encoding the regulatory Smads, Smad2 or Smad3, were co-transfected with full-length CTGF promoter/SEAP constructs into NIH/3T3 fibroblasts (Figure 3.12A) and HFF (Figure 3.12B) cells in the presence or absence of the co-Smad, Smad4, and reporter gene activity assessed. Individually, Smad3 but not Smad2 increased (1.9 and 1.1 fold respectively) promoter activity of CTGF800 compared to the CMV control plasmid in the absence of exogenous TGF- β 1 in NIH/3T3 cells. Transfection of Smad4 alone induced CTGF promoter activity 2.4 fold (Figure 3.12A). Smad3/4 significantly enhanced ($P<0.05$) CTGF promoter activity 3.2 fold in NIH/3T3 cells (Figure 3.12A). Whereas co-transfection of Smad2/4 had no effect, Smad3/4 significantly ($P<0.02$) potentiates CTGF promoter activity in response to TGF- β 1 compared to Smads2, 3 and 4 alone (Figure 3.12A). In support of these observations HFF cells exhibited a similar response, with Smad3/4 significantly enhancing ($P<0.05$) CTGF800 reporter activity (Figure 3.12B). These results suggest that Smad3 and Smad4 can transactivate CTGF in both NIH/3T3 and HFF cells.

The efficacy of the Smad expression vectors was confirmed by the transactivation of their respective DNA binding consensus sequences linked to a luciferase reporter gene. Co-transfection of Smad2/Smad4 induced an 8 fold increase in basal and 4 fold increase in TGF- β 1 induction of the Smad2 reporter construct ARE, compared to CMV control vector alone. No significant response to Smad3 and Smad4 was found (Figure 3.12C). Conversely, Smad3/Smad4 potently induced reporter gene basal (6 fold) and TGF- β 1 induced (5 fold) expression of the Smad3 construct CAGA (Figure 3.12D). No significant effect by Smads 2, 3 and 4 or TGF- β 1 treatment was noted on the background reporter vector, pSEAPbasic, in NIH/3T3 cells (Figure 3.10E).

A



B



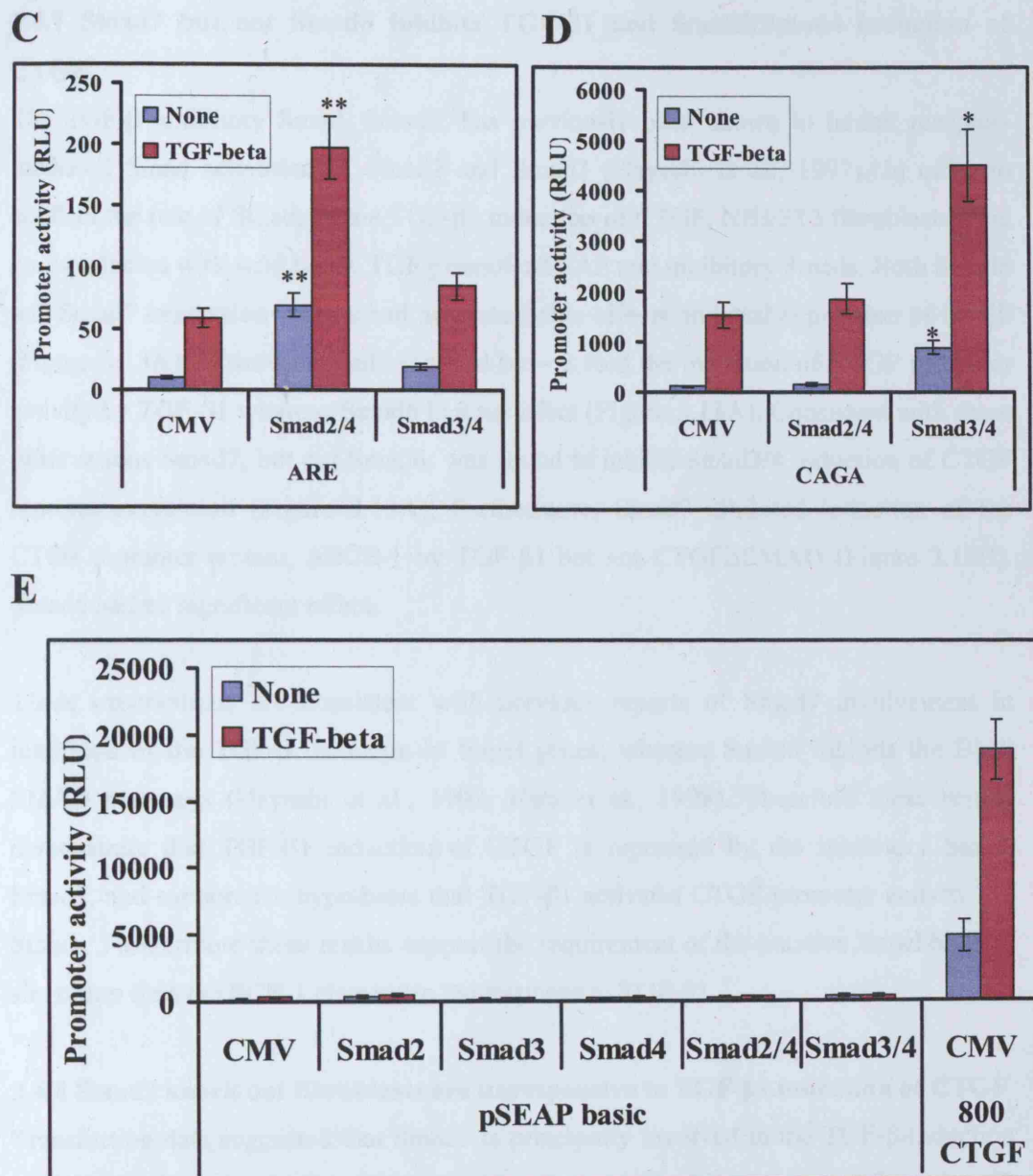


Figure 3.12

Transactivation of the CTGF promoter by Smads.

CTGF800 promoter reporter construct was transiently transfected with either Smad 2 or Smad3 in the presence or absence of Smad4 and/or TGF- β 1 (2ng ml⁻¹) in NIH/3T3 (A) and HFF (B) cells. SEAP promoter reporter activity was assessed, and the relative light units (RLU) based on SEAP expression (25 μ l of conditioned medium) normalised to β -galactosidase activity from a co-transfected CMV- β -galactosidase plasmid are shown (mean \pm SEM, n=6). Transactivation of ARE (C) or CAGA (D) reporter constructs by Smad2/4 or Smad3/4 was assessed and RLU based on luciferase expression normalized to β -galactosidase activity from a co-transfected CMV- β -galactosidase plasmid are shown (mean \pm SEM, n=3). Effects of Smad2, Smad3 and Smad4 on pSEAP basic activity (E) were assessed in the presence or absence of TGF- β 1 (2ng ml⁻¹) (mean \pm SEM, n=3); *p<0.05, **p<0.02 Students unpaired t-test.

3.4.7 Smad7 but not Smad6 inhibits TGF- β 1 and Smad3/Smad4 induction of CTGF.

The TGF- β inhibitory Smad, Smad7, has previously been shown to inhibit receptor-mediated Smad activation of Smad2 and Smad3 (Hayashi et al., 1997). In order to confirm the role of Smads in the TGF- β 1 induction of CTGF, NIH/3T3 fibroblasts were co-transfected with wild type CTGF promoter/SEAP and inhibitory Smads. Both Smad6 and Smad7 expression vectors had no discernable effects on basal expression of CTGF (Figure 3.13A). However Smad7 reduced by ~ 2 fold the induction of CTGF promoter activity by TGF- β 1 whereas Smad6 had no effect (Figure 3.13A). Consistent with these observations Smad7, but not Smad6, was found to inhibit Smad3/4 induction of CTGF reporter expression (Figure 3.13A). Furthermore, Smad7 inhibited induction of the CTGF promoter mutant, Δ BCE-1 by TGF- β 1 but not CTGF Δ SMAD (Figure 3.13B). Smad6 had no significant effect.

These observations are consistent with previous reports of Smad7 involvement in inhibition of the TGF- β induction of target genes, whereas Smad6 inhibits the BMP SMAD pathways (Hayashi et al., 1997; Hata et al., 1998). Therefore these results demonstrate that TGF- β 1 induction of CTGF is repressed by the inhibitory Smad, Smad7, and support the hypothesis that TGF- β 1 activates CTGF promoter activity via Smads. Furthermore these results support the requirement of the putative Smad binding site rather than the BCE-1 element in the response to TGF- β 1.

3.4.8 Smad3 knock out fibroblasts are unresponsive to TGF- β 1 induction of CTGF

Transfection data suggested that Smad3 is principally involved in the TGF- β -induction of CTGF (Figure 3.14). To verify this, we obtained fibroblasts cultured from Smad3 knockout and wild-type foetal mice and determined their response to TGF- β 1 treatment (Yang et al., 1999). Both Smad3 null and wild-type cells were treated in the presence and absence of TGF- β 1 and CTGF mRNA and protein levels determined at six or twenty four hours respectively. Both wild type and knockout embryonic mouse fibroblasts displayed high constitutive levels of CTGF mRNA and protein, as determined by real time PCR and Western blot (Figure 3.14 A and B). This result is consistent with several studies that demonstrate constitutive expression of CTGF protein to be serum responsive. In contrast to basal expression of CTGF, wild type fibroblasts showed a marked induction of CTGF mRNA expression at six hours (8 fold) and protein expression (3 fold) in response to TGF- β 1 (Figure 3.14 A and B).

Conversely Smad3 null fibroblasts showed a significantly reduced response to TGF- β 1 treatment induction of CTGF mRNA (2.5 fold) and protein (1.1 fold) (Figure 3.14 A and B). Consistent with this, adult dermal Smad3 null fibroblasts showed a marked reduction in CTGF induction in response to TGF- β 1 treatment (Figure 3.14 B right panel)

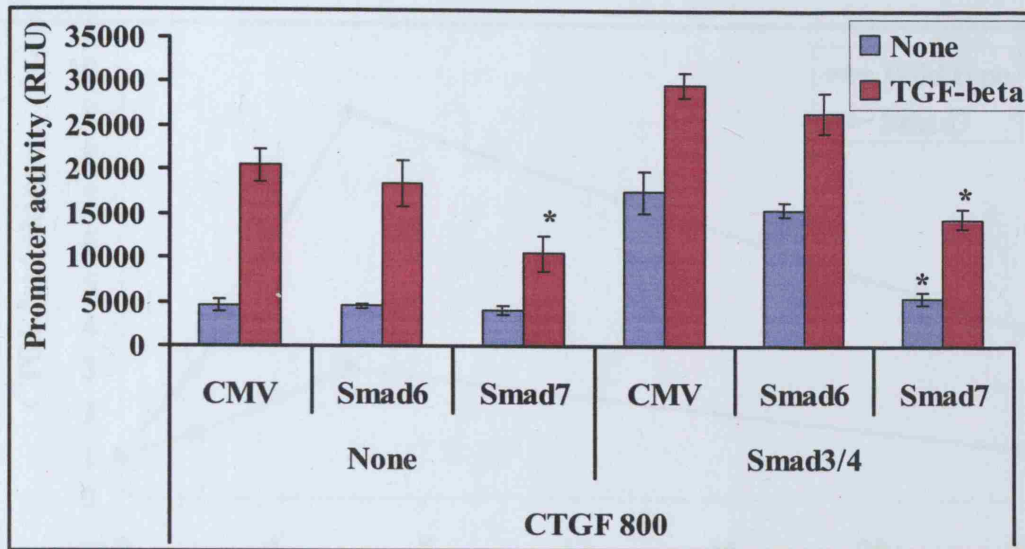
In support of these observations, wild type but not Smad 3 null fibroblasts transfected with the CTGF promoter/SEAP construct showed a significant ($P < 0.05$) response to TGF- β 1 treatment. Further rescue of the Smad3 null fibroblast by co-transfection with Smad3 significantly ($P < 0.05$) restored TGF- β 1 induction of the CTGF promoter/SEAP construct (Figure 3.14C). These observations further support the requirement of Smad3 in TGF- β 1 induction of CTGF expression.

3.4.9 Smad 3 and Smad 4 form a complex on the CTGF promoter

To determine whether Smad proteins could bind to the putative Smad binding site, we performed an electrophoretic mobility shift assay (EMSA) with NIH/3T3 fibroblast nuclear extract, made from cells that had been treated with TGF- β 1 for one hour. A radiolabeled double-stranded oligonucleotide probe containing the putative SMAD binding site was used as a probe (CGAGCTTTTTCAGACGGAGGAATG CTGAGTGTCA). Three protein complexes bound to the probe (Figure 3.15 arrows). Binding was specific, as the DNA protein interactions could be competed by adding excess unlabeled oligonucleotide (Figure 3.15; lanes 3-4). Binding of one of the complexes could be abolished by pre-incubating nuclear extract with an anti-Smad3 (lane 8) or anti-Smad4 antibody (lane 10), but not with an anti-Smad2 antibody (lane 6), indicating that the protein complex formed on this region of DNA contained Smad3 and Smad4 but not Smad2. No complex was formed between antibody and probe alone (Figure 3.15; lanes 5, 7 and 9).

Taken together, these data suggest that the region of DNA containing the putative Smad binding site identified as being important for gene expression contains a Smad binding site, able to interact with Smad3 and Smad4, and is important for the induction of TGF- β 1 induction of CTGF.

A



B

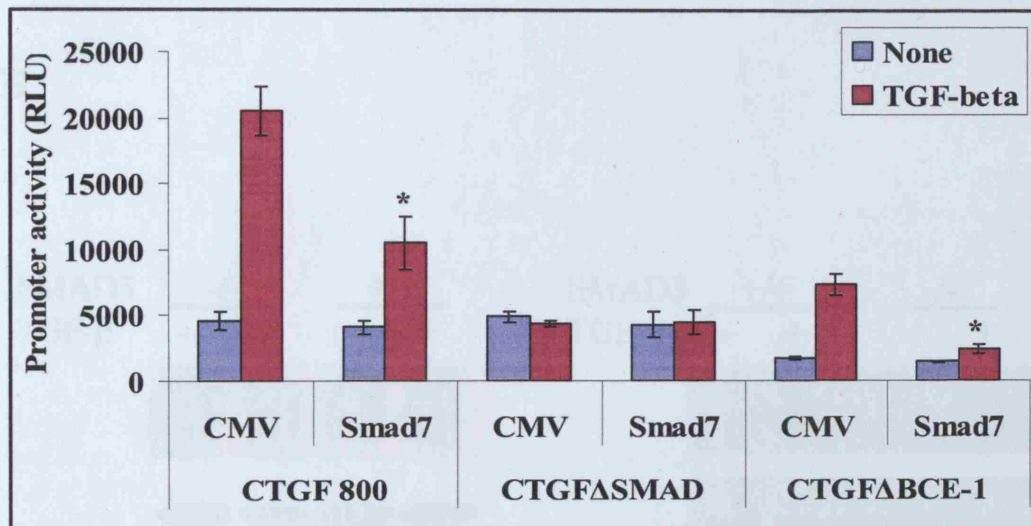
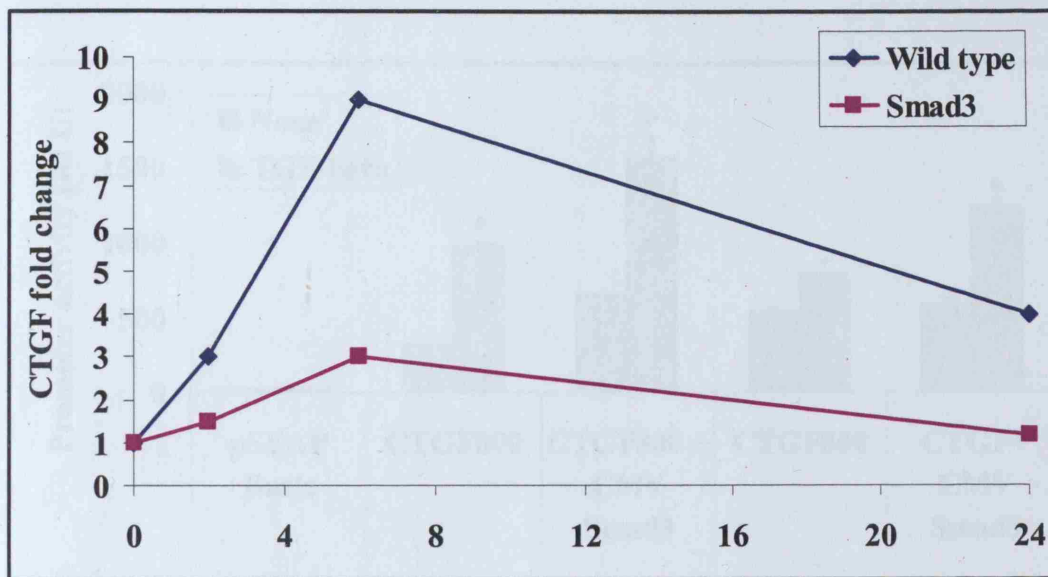


Figure 3.13

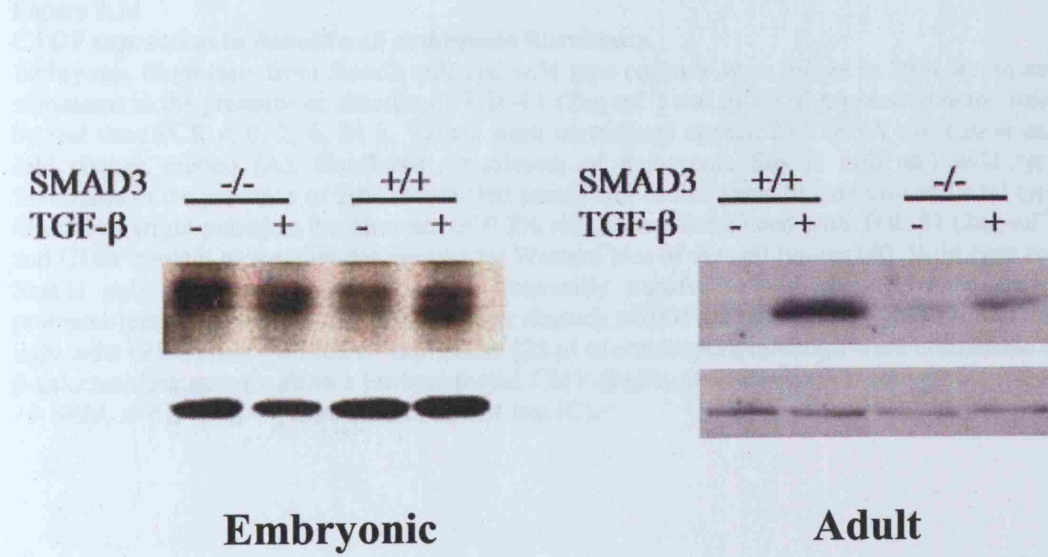
Smad7 inhibits Smad3/Smad4 and TGF- β 1 induction of CTGF via the Smad binding site.

In order to confirm the effect of inhibitory Smads (Smad6 and Smad7), NIH/3T3 were co-transfected with CTGF full length reporter promoter construct (CTGF800) in the presence or absence of Smad6 or Smad 7, with or without Smad3 and Smad4, and SEAP activity assessed after 24h \pm TGF- β 1 (2ng ml⁻¹) (A). CTGF800, CTGF Δ SMAD or CTGF Δ BCE-1 was co-transfected with control (CMV) or Smad7 expression vector in the presence of absence of TGF- β 1 (2ng/ml). CTGF promoter activity was determined in RLU based on SEAP expression (25 μ l of conditioned medium) and normalized to β -galactosidase activity from a co-transfected CMV- β -galactosidase plasmid (mean \pm SEM, n=6); *p<0.05, Students unpaired t-test (B).

A



B



C

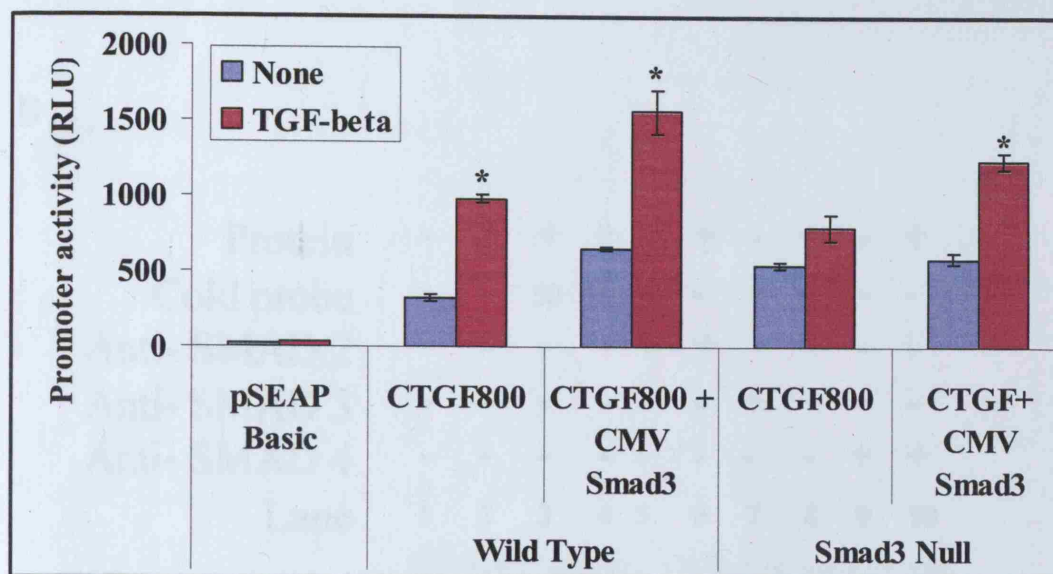


Figure 3.14

CTGF expression in Smad3 null embryonic fibroblasts.

Embryonic fibroblasts from Smad3 null and wild type controls were grown in 20% serum and stimulated in the presence or absence of TGF- β 1 (2ng ml⁻¹) and mCTGF expression determined by real time PCR at 0, 2, 6, 24 h. Values were normalized against 28S rRNA expression and fold change plotted (A). Confluent monolayers of embryonic Smad3 null and wild type fibroblasts in the presence of 20% serum (left panel) and adult dermal Smad3 null and wild type fibroblasts (right panel) in the presence of 0.2% serum were stimulated with TGF- β 1 (2ng ml⁻¹) and CTGF protein expression determined by Western blot of the cell lysates (B). Wild type and Smad3 null embryonic fibroblasts were transiently transfected with the CTGF800 SEAP promoter/reporter construct in the presence or absence of TGF- β 1 and/or CMV Smad3. Relative light units (RLU) based on SEAP expression (25 μ l of conditioned medium) were normalized to β -galactosidase activity from a co-transfected CMV- β -galactosidase plasmid are shown (mean \pm SEM, n=6); *p<0.05, Students unpaired t-test (C).

A

TGCCAGCTTTTTCAGACGGAGGAATGCTGAGTG

SMAD site

B

Protein	-	+	+	+	-	+	-	+	-	+
Cold probe	-	-	50	100	-	-	-	-	-	-
Anti- SMAD 2	-	-	-	-	+	+	-	-	-	-
Anti- SMAD 3	-	-	-	-	-	-	+	+	-	-
Anti- SMAD 4	-	-	-	-	-	-	-	-	+	+
Lane	1	2	3	4	5	6	7	8	9	10

SMAD 3 →
→
→

**Free
Probe**



Figure 3.15

Smad3 and Smad4 bind the CTGF promoter region at -244bp.

Electrophoretic mobility shift analysis was performed on a 33 bp double stranded oligomer probe containing the putative Smad binding site of the CTGF promoter (A) with 5ug of nuclear extracts from NIH/3T3 cells treated for 1 h with TGF- β 1 (2ng ml⁻¹) (B). A complex was observed (arrow) that was specific, as 50 or 100-fold excess of cold competitor competed for protein binding to radio labeled DNA (lanes 3 and lanes 4). Pre-incubation of the nuclear extracts before the addition of the probe with anti-Smad2 (lane 6) did not effect binding whereas anti-Smad3 (lane 8) or anti-Smad4 (lane 10) inhibited binding of the Smad complex (B).

3.5 Discussion

In this chapter the role of Smads in the induction of CTGF by TGF- β in dermal fibroblasts was investigated. The potent and rapid induction of CTGF by TGF- β in fibroblasts has been reported by several groups (Shi-wen et al., 2000; Grotendorst et al., 1996; Wahab et al., 2001b). In order to delineate the mechanism of CTGF induction by TGF- β we initially wished to clarify the parameters of endogenous transcriptional and reporter gene response in several fibroblast cell lines. In this study CTGF mRNA in unstimulated fibroblasts was present at low or un-detectable levels. Previous reports have shown *in vitro* induction of CTGF by TGF- β 1 and - β 2 (Grotendorst et al., 1996; Leask et al., 2003). In keeping with this we show in addition to TGF- β 1 and -2, TGF- β 3 induces endogenous CTGF expression *in vitro*. Interestingly TGF- β 2 induced maximal CTGF mRNA induction at two fold higher concentrations compared to TGF- β 1 and - β 3 (Figure 3.4). This may reflect differences in the bioactivity of the recombinant material or a difference in cellular response to TGF- β 2; for example, TGF- β 2 requires the accessory receptor beta-glycan for high affinity binding (Lopez-Casillas et al., 1993). Although *in vivo* differences in bioactivity between TGF- β isoforms have been reported both in the induction of genes, such as collagen (COL1A2), and in the function (Kinbara et al., 2002) – e.g. TGF- β 3 has been shown to promote scarless wound healing in contrast to TGF- β 1 (Ferguson and O'Kane, 2004) - *in vitro* all three isoforms in this study appear to function in a similar manner.

To investigate the mechanism of CTGF induction by TGF- β and the role of Smads, a 4.5 kb upstream region of the CTGF promoter was cloned into the pGL3 luciferase reporter vector and compared to a smaller promoter construct containing 800 bp of the non-coding regulatory region of CTGF, 5' to the TATA box. Transient transfection of both CTGF promoter/reporter constructs responded to TGF- β 1 in a dose dependent manner. In keeping with endogenous CTGF mRNA responses, all three TGF- β isoforms induced reporter gene activity (Figure 3.7). Based upon these observations the decision was made to use TGF- β 1 for the remainder of the studies.

During the course of studying the CTGF promoter/reporter constructs with respect to the response towards two known inducers of CTGF expression, thrombin and ET-1, both were shown to activate CTGF reporter activity (Figure 3.8). Induction of CTGF by ET-1 was mediated via elements within the first 800 bp, whereas thrombin was only able to induce reporter gene activity in the larger construct. This suggests the thrombin

response element in the CTGF promoter is distinct from the TGF- β response element contained within the first 800 bp. Thrombin is known to activate several signalling pathways including PKC, the MAP kinases such as JNK, and NF κ B (Pontrelli et al., 2004; Tantivejkul et al., 2005). Motif scanning of CTGF promoter for a recently identified thrombin response element in the human platelet-derived growth factor B-chain (Scarpati and DiCorleto, 1996), CCACCC, identified one such site between -4500 and -800 bp. Mapping the signalling pathways required for induction of CTGF by thrombin falls outside the scope of these studies and remains unknown. These experiments identify and extend the observation by Grotendorst *et al.* of a TGF- β response element lying in the first 800 bp of the CTGF promoter (Grotendorst et al., 1996).

Initially to map the TGF- β response region in the CTGF promoter a deletion series of the CTGF promoter linked to a reporter gene was transiently transfected into human foreskin fibroblasts and NIH/3T3 and assessed. In contrast to a report by Grotendorst *et al.* the CTGF promoter construct -166 to +17 bp containing the previously reported TGF- β response element, TRE, was unresponsive to TGF- β 1 stimulation. Rather TGF- β induction of the CTGF promoter/reporter construct was confined to elements 13 bp upstream of this region between -244 and -166 bp of CTGF TATA box. Sequence inspection of this region confirmed a putative Smad binding site sharing 75% homology to the previously reported Smad3 response element (Zawel et al., 1998).

Point mutations in this putative Smad binding element led to the constructs being non-responsive towards TGF- β . In contrast, mutation of the TRE element did not alter the induction by TGF- β but rather caused a significant decrease in the basal expression of the promoter construct. This led to the identification and reassignment of this element as a basal control element or BCE-1. Initial identification of the TRE element had shown proteins binding to this region to be constitutively present in fibroblast nucleoplasm (Grotendorst et al., 1996), whereas Smads are primarily cytosolic and translocate to the nucleus on TGF- β activation (Dong et al., 2000; Liu et al., 1997). Recent CTGF promoter deletion mapping studies by Shi-wen *et al.* have shown ET-1 to induce CTGF expression through this element (Xu et al., 2004). In silico analysis of this region for known transcription factor binding sites indicates the presence of an Nkx2.5 transcription factor binding motif with 83% homology to consensus Nkx2.5 binding site. Interestingly ET-1 has been reported to induce Nkx2.5 mRNA expression in

cardiac muscle cells (Takebayashi-Suzuki et al., 2001). Together, these data indicate the induction of CTGF expression by TGF- β to be mediated via promoter regions between -244bp and -166bp containing a putative binding site, while the previously identified site controls basal expression and the response to ET-1.

The role of Smads in the induction of CTGF by TGF- β was also clearly shown by transfection experiments involving different components of the Smad signalling pathway (Figure 3.12). Consistent with the CTGF expression being regulated by Smad3, promoter/reporter activity was induced by Smad3 and required an intact putative Smad binding site but not a BCE-1 site. In support of Smad3 mediating TGF- β induction, the expression of endogenous CTGF in embryonic Smad3 null fibroblasts was determined. These data (Figure 3.14) demonstrate that the absence of Smad3 severely affects the ability of TGF- β to induce CTGF (Ryseck et al., 1991). Consistent with this, Smad3 null adult dermal fibroblasts exhibit impaired induction of CTGF by TGF- β . Studies by the Roberts group have subsequently confirmed this observation (Flanders et al., 2003). Recent gene profiling studies indicate Smad3 to be an essential mediator of TGF- β signalling, directly activating transcription through Smad3/Smad4 DNA-binding motifs that are characteristic for immediate-early target genes that encode regulators of transcription, and signal transducers. Conversely, Smad2 is predominantly involved in the transmodulated regulation of both immediate-early and intermediate genes by TGF- β /Smad3 (Yang et al., 2003). Furthermore, TGF- β and Smad3 mediated induction of CTGF is repressed by the actions of the inhibitory Smad, Smad7 (Figure 3.13) and the antagonists of TGF- β /Smad signalling Ski and SnoN (Holmes et al., 2001). Thus the role of Smad3 in the induction of the immediate early gene, CTGF, is consistent with Smad3 mediating TGF- β effects on this immediate-early gene.

Induction of CTGF transcription by TGF- β is rapid, occurring within the first hour *in vitro*. In order to determine whether the induction of CTGF by Smads was indeed a direct result of Smad interaction with the Smad promoter element or a secondary effect, EMSA using a region of DNA spanning the Smad binding site within the CTGF promoter as a target was performed. EMSA analysis identified 3 complexes which formed on a radiolabelled double strand oligomer comprising 33 bp of the CTGF promoter and spanning the putative Smad binding site. Incubation with antibodies against Smad3 and Smad4, but not Smad2, resulted in a marked loss of one such complex, presumably through steric hindrances of Smads to binding to DNA. Thus

Smad 3 and Smad 4 are able to directly interact with a small region of TGF- β encompassing the entire Smad binding site. In these studies I have identified an element within the CTGF promoter through which TGF- β induces CTGF expression in a predominantly Smad3 dependent manner in HFF and NIH/3T3 fibroblasts. In addition the previously identified TGF- β element appears to control the basal activity of the CTGF promoter in fibroblasts, rather than any primary involvement in response towards TGF- β , although this does not preclude it from functioning in this manner in cell types other than those currently investigated here.

Studies by other groups have subsequently confirmed the requirement of Smads in the activation of CTGF by TGF- β in several cell types, including renal proximal tubule epithelial cells (Phanish et al., 2005), smooth muscle cells (Fu et al., 2001) and mesangial cells (Chen et al., 2002). In this and other laboratories the activation of non-Smad signalling pathways, including the MAP kinases, has been demonstrated to be critical in the TGF- β mediated induction of CTGF (Fu et al., 2001; Leask et al., 2003; Phanish et al., 2005). The MAP kinases, such as ERK are known to have both positive and negative effects on Smads. Indeed ERK activation by TGF- β in hepatic stellate cells promotes Smad nuclear translocation, through the phosphorylation of the linker region of Smad3 (Hayashida et al., 2003). The precise mechanism of action in fibroblasts remains unknown.

TGF- β /Smad induction of genes often require the actions of co-factors, such as Sp1 and p300 (Ihn et al., 2006; Janknecht et al., 1998; Nishihara et al., 1998). Although induction of CTGF by TGF- β is independent of p300 (Holmes et al., 2001), subsequent studies have identified an additional element required for the induction of CTGF by TGF- β (Leask et al., 2003). Recent studies have shown members of the ETS family of transcription factors to bind this element and synergise with Smad3 to promote the induction of the CTGF promoter by TGF- β 1 (Nakerakanti et al., 2006; Van Beek et al., 2006).

3.6 Summary

In conclusion, the studies presented within this chapter have shown that all three TGF- β isoforms induce CTGF expression, via elements in the proximal portion of the CTGF promoter. Also, in addition, ET-1 and thrombin, acting via discrete elements in proximal and distal portions of the CTGF promoter, induce CTGF promoter activity. The formerly characterised TGF- β response element acts, in NIH/3T3 and HFF cells, as a regulator of basal expression, and is not essential for TGF- β induction of CTGF. Instead TGF- β induces CTGF expression via a Smad3 complex binding to a *bona fide* SMAD transcription factor binding site.

Chapter 4

TGF- β /Smad regulation of CTGF in SSc dermal fibroblasts

4.1 Introduction

The most persuasive evidence for the role of TGF- β in fibrosis comes from animal models such as bleomycin induced lung injury (Coker et al., 1997) or the murine GvHD model (Zhang et al., 2002) discussed in Chapter 1, Section 1.3. Targeted disruption of TGF- β or specific Smads leads to a reduction in fibrosis (Bonniaud et al., 2005; Nakao et al., 1999). Given the apparent pivotal role of TGF- β to modulate fibrosis it is not surprising that much attention has focused on TGF- β in SSc. LeRoy postulated in the early 1990s that TGF- β was a causative agent of SSc (Smith and LeRoy, 1990). The evidence, however, for a role of TGF- β in SSc is somewhat contentious and contradictory (Chapter 1; 1.2.7.3). With circumstantial data to support the role of TGF- β in the onset of fibrosis in SSc, its precise role in the maintenance of the 'fibrogenic' phenotype of SSc remains unclear. The absence of TGF- β expression and the cellular 'memory' of TGF- β by SDF (discussed 1.2.7.2) have led some researchers to seek downstream modulators of TGF- β fibrotic actions. One such factor known to influence ECM synthesis is CTGF (Shi-wen et al., 2000). The mechanism by which CTGF over-expression in SSc is sustained is currently unknown. In the previous chapter I demonstrated that TGF- β potently induced CTGF expression in normal fibroblasts in a Smad dependent manner. This led to the hypothesis that excessive TGF- β expression and/or Smad signalling, may lead to dysregulated expression of CTGF in SSc fibroblasts and thus contribute to maintaining the fibrogenic phenotype of these cells.

4.2 Aims

In the early 1970s LeRoy demonstrated that SDF cultured *in vitro* maintain their activated appearance and excessive production of type I collagen, compared to healthy controls, for a number of passages (LeRoy, 1972; LeRoy, 1974). During the intervening years several groups have observed that explanted fibroblasts maintain their 'fibrogenic' phenotype including the expression of CTGF (Xu et al., 1998b; Gruschwitz et al., 1992; Shi-wen et al., 2000). In the previous chapter CTGF expression was shown to be potently induced by TGF- β in a Smad3 dependent manner. Using dermal fibroblast cell lines established from SSc patients and healthy age matched controls the following questions were addressed:

- 1- Is *in vitro* CTGF over-expression in SDF TGF- β dependent?
- 2- Is CTGF over-expression in SDF due to dysregulation in Smads?
- 3- What is the minimal region of the CTGF promoter required to confere/exhibit elevated expression in SDF?

4.3 Material and Methods

4.3.1 Cell culture

4.3.1.1 Cell lines

All primary dermal fibroblast cells were maintained as described in Section 2.2.1. For experiments, cells were used between passage 2 and 5. Mink lung epithelial cells (ML) containing the PAI-1 promoter luciferase construct, the generous gift of Dr Daniel Rifkin (Rifkin, 2005), transformed with a TGF- β responsive portion of the PAI-1 promoter linked to the reporter gene firefly luciferase were maintained in FGM media supplemented with 250 $\mu\text{g ml}^{-1}$ of Geneticin (G-418 sulphate) (Sigma, UK).

4.3.1.2 Fibroblast conditioned media

Control and SSc dermal fibroblast cell lines were maintained in FGM media until fully confluent. Media was replaced by DMEM (Invitrogen, UK) containing 0.1% BSA (w/v) (Sigma, UK) supplemented with L-glutamine, penicillin G (100 U/ml) and streptomycin sulphate (100 $\mu\text{g ml}^{-1}$) (Invitrogen, UK) and the conditioned media (CM) collected 24 h later. CM was divided into two, and one half heated at 80 °C for 10min to convert all latent TGF- β into the active form. Medium was stored at -70 °C.

4.3.1.3 Mink lung Assay

The mink lung assay for the measurement of bioactive TGF- β was performed as described by Abe *et. al* (Abe et al., 1994). Briefly, sub- confluent ML cells were seeded at a density of 1.6×10^4 cells per well of a 96-well tissue culture plate (Costar, UK) and were allowed to attach for 3 h at 37°C in a 5% CO₂ incubator (Abe et al., 1994). The medium was replaced with 100 μl CM, heat-activated CM from fibroblasts, or media alone, in the presence of 10 $\mu\text{g ml}^{-1}$ of neutralizing-anti-TGF- β antibody 1D11 (R&D systems, UK) or mouse IgG (Santa Cruz, UK) and ML cells maintained for 18 h at 37°C in a 5% CO₂ incubator. Cell extracts were prepared and PAI-1 promoter activity determined by luciferase RLU as described in Section 2.2.4.3. CM media from control, and SDF lines were assessed for TGF- β activity in triplicate. Active levels of TGF- β were defined as the difference in RLU between 1D11 treated and IgG treated CM, whereas latent TGF- β was defined as the difference between total RLU induced by heat-activated CM in the presence of IgG and 1D11. All values are represented as the RLU less the endogenous activity in control media on the PAI-1 luciferase promoter (22 RLU \pm 6).

4.3.2 Protein expression

4.3.2.1 Antibodies

All antibodies unless stated otherwise were used as described in Section 2.4.4. Neutralizing TGF- β antibody, 1D11 (R&D systems) recognises bovine, mouse and human TGF- β 1 and TGF- β 2. Recognition of TGF- β 3 has been characterized in chicken. Neutralization was confirmed by the manufacturer in the mink lung epithelial assay (Abe et al., 1994).

4.4 Results

4.4.1 CTGF expression in control and SDF cell lines

Previously, Shi-wen *et al.* have shown cultured SDF cell lines established from areas of fibrosis maintain their expression of CTGF for a number of passages (Shi-wen *et al.*, 2000). SSc is characterised by fibrosis and general over expression of collagen type I, but not all SSc fibroblast lines exhibit this phenotype in culture (Kessler-Becker *et al.*, 2004). Thus the following experiments were aimed at identifying a series of SDF cell lines with elevated CTGF expression on which the remainder of the studies would be conducted.

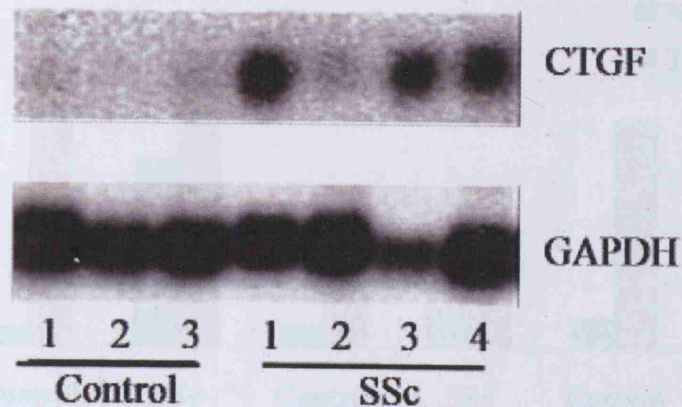
Investigation of CTGF mRNA levels, as determined by northern blot (Figure 4.1A), and protein levels, as confirmed by Western blotting gel electrophoresis (Figure 4.1B), showed that only one of the six normal cell lines, compared to thirteen of the fifteen SDF lines screened, exhibited any degree of CTGF expression. mRNA over-expression correlated with expression of CTGF in the cell monolayer. In keeping with the notion of CTGF being a robust and potentially useful marker of fibrosis, in this study over 80 percent of the SDF cell lines screened demonstrate elevated expression of CTGF.

4.4.2 Response of SSc fibroblasts to TGF- β stimulation

The precise role of TGF- β in SSc still remains unknown with contradictory reports concerning the requirement for, and effect of, this growth factor in SSc. In order to investigate this further I determined the response, as measured by expression of CTGF and two other known TGF- β response genes, Collagen Ia2 (COL1A2) and plasminogen activator inhibitor (PAI-1), to exogenous addition of TGF- β to SDF lines. Using a dose of 2ng ml⁻¹ TGF- β 1, SSc and control lines were incubated for 8 hours in the presence or absence of TGF- β , and CTGF mRNA levels assessed by real time PCR. The results of this study demonstrate SDF exhibit a higher un-stimulated level of expression of CTGF, COL1A2 and PAI-1 compared to the control cells. However, whereas control cells demonstrated a strong response to TGF- β 1 stimulation, inducing a twenty fold, a three fold and fourteen fold increase in mRNA of CTGF, COL1A2 and PAI-1 respectively, SDF appeared to respond more modestly (Figure 4.2A). Induction in response to TGF- β by SDF of CTGF, COL1A2 and PAI-1 was 1.4, 1.5 and 1.8 fold respectively, suggesting that SDF are transcriptionally refractory to exogenous addition of TGF- β 1.

To seek support of this observation, the effect of TGF- β 1 on protein levels of CTGF, PAI-1 and type I collagen was determined. Two SSc cell lines previously screened as high CTGF producers exhibited higher basal expression of PAI-1, and collagen type I protein levels compared to control cell lines as assessed by Western blot (Figure 4.2B). Control fibroblast cell lines showed a strong induction of PAI-1 and CTGF and a more modest induction of collagen type I protein expression in response to TGF- β 1 as determined by densitometry analysis. SDF which exhibited a higher basal expression of these genes did not respond to further TGF- β 1 treatment (Figure 4.2B).

A



B

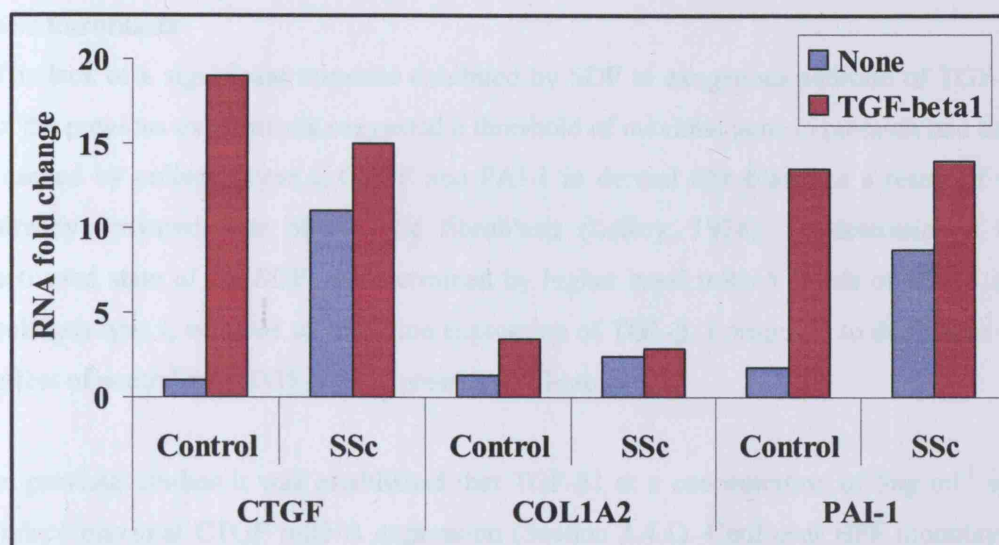


Figure 4.1

Northern and Western blot of CTGF expression in control and SSc cell lines.

Control and SSc cell lines were grown to confluence and incubated in 0.1% serum over-night. RNA was isolated and 5µg of total RNA probed for CTGF levels by northern blot and loading controlled for by GAPDH expression levels (A). Total protein from the monolayers of confluent control and SSc cell lines was isolated and 10µg protein separated by gel electrophoresis with CTGF levels determined by immunoblotting analysis using an anti-CTGF specific antibody. Total protein levels were normalised using a monoclonal specific antibody against actin. (B). Phospho-images of representative northern and Western blots are shown.

A



B

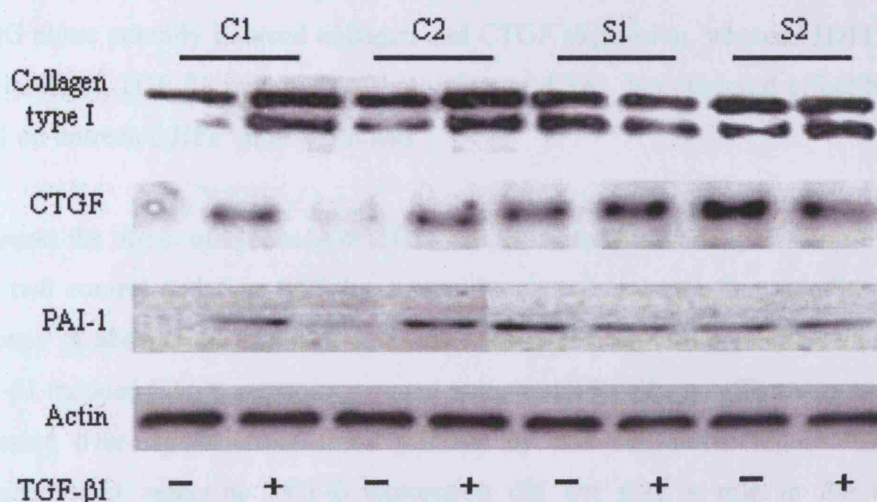


Figure 4.2

Endogenous gene responses to TGF-β1 stimulation by SSc and control cell lines.

Three Control and SDF cell lines were grown to 80% confluence and serum starved for 24 h. Following stimulation with 5ng ml⁻¹ of TGF-β1 for 8 h total RNA was isolated from the fibroblast monolayers and RNA pooled. Real time PCR was performed for CTGF, COL1A2, PAI-1 and ribosomal 28S, by Dr Sarah Howat (King's College London) and gene expression was normalised to 28S levels. Fold change was based on the change of normalised levels of CTGF, COL1A2 or PAI-1 compared to basal expression of these genes in the control cell lines (A). Two control and four SDF lines were serum starved and stimulated for 24 h in the presence of 5ng ml⁻¹ TGF-β1. Western blot was performed on media and cell monolayers and levels of collagen type I, CTGF and PAI-1 levels determined. Loading was controlled for by actin expression (B). Protein expression levels were semi-quantified as described in Chapter 2, Section 2.6.

4.4.3 Effect of inhibition of TGF- β on CTGF and collagen type I over expression in SSc fibroblasts

The lack of a significant response exhibited by SDF to exogenous addition of TGF- β 1 in the previous experiments suggested a threshold of maximal gene expression had been reached by collagen type I, CTGF and PAI-1 in dermal fibroblasts as a result of the already activated state of the SSc fibroblasts (LeRoy, 1974). To determine if the activated state of the SDF, as determined by higher basal mRNA levels of CTGF and collagen type I, was due to autocrine expression of TGF- β , I proposed to determine the effect of neutralizing TGF- β on expression of these genes.

In previous studies it was established that TGF- β 1 at a concentration of 5ng ml⁻¹ will induce maximal CTGF mRNA expression (Section 3.4.1). Confluent HFF monolayers were incubated with the murine pan-specific TGF-beta-neutralizing monoclonal antibody, 1D11, or an IgG control antibody in the presence or absence of TGF- β 1 and were assessed for CTGF and type I collagen protein expression. TGF- β 1 in the presence of IgG alone potently induced collagen and CTGF expression, whereas 1D11 at 10 μ g ml⁻¹ inhibited TGF- β 1 induction of both (Figure 4.3A). No observed effect by IgG or 1D11 on untreated HFF cells was noted.

To assess the effect of blockade of TGF- β on constitutive over-expression of CTGF in SSc, two control and four SDF lines were incubated in serum free conditions in the presence or absence of 1D11 or IgG. Whereas 1D11 showed a marked inhibition of TGF- β 1 induced CTGF and collagen expression in HFF cells, no effect was noted in the excessive over-expression of these proteins by SSc cell lines (Figure 4.3B). This suggested that autocrine TGF- β expression did not play a role in the excessive expression of these genes in explanted SDF.

4.4.4 Measurement of TGF- β expression by SSc fibroblasts

TGF- β is synthesised and secreted as a biologically inactive or 'latent' complex. Activation of this complex leads to the release of the mature and biologically active form of TGF- β (Rifkin, 2005). In previous studies I have shown over expression of CTGF and collagen type I by the SDF is unaffected by inhibition of TGF- β (Figure 4.3). To confirm this observation I proposed to determine the biological activity of active and latent TGF- β in conditioned media from fibroblasts, using the functionally relevant PAI-1 promoter linked to the luciferase reporter gene stably integrated into mink lung epithelial cells (Figure 4.4).

PAI-1 promoter activity, as measured by luciferase relative light units (RLU), demonstrated a linear dose response to recombinant TGF- β up to 5ng ml⁻¹. Incubation with 10 μ g ml⁻¹ 1D11 completely blocked induction (Figure 4.4A). Visual assessment of cells incubated with TGF- β 1 doses higher than 5ng ml⁻¹ suggested reduced proliferation of ML cells.

Conditioned media (CM) from the control cell lines significantly ($p < 0.02$) induced PAI-1 promoter activity (390 ± 45 RLU) in the presence of IgG alone. Incubation with 1D11 significantly ($p < 0.05$) reduced luciferase activity by around 50%; (210 ± 39 RLU), suggesting half of the PAI-1 promoter activity was attributable to secreted active TGF- β . Analogous to these observations, SSc CM exhibited a similar effect on PAI-1 reporter promoter (430 ± 78 RLU) of which 50% of the PAI-1 activity was inhibited by 1D11 (197 ± 43 RLU). This suggests that active TGF- β levels, as determined by PAI-1 promoter activity, were similar in both control and SSc cell lines. In contrast SDF secreted three fold more latent TGF- β compared to control cells: 430 ± 220 RLU versus 150 ± 12 RLU respectively ($p < 0.02$) (Figure 4.4).

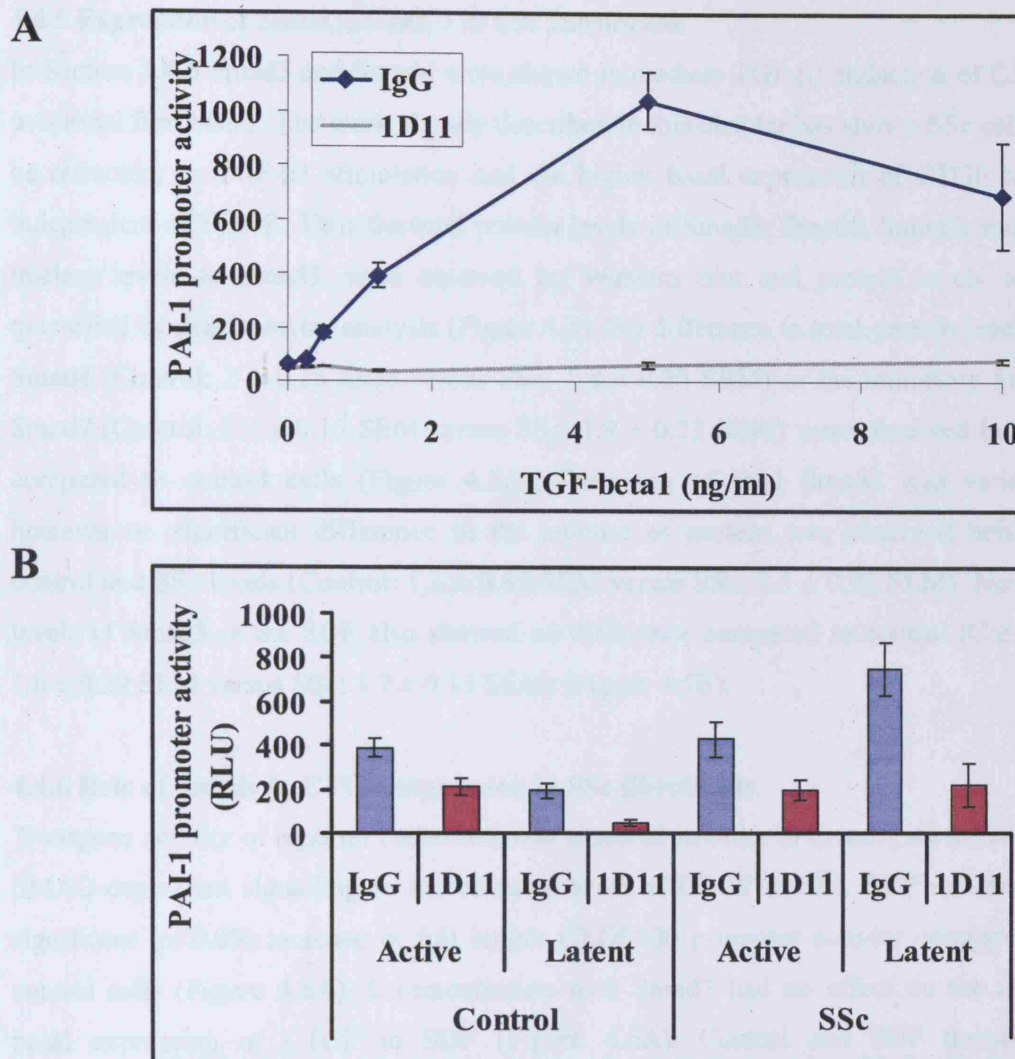


Figure 4.4

PAI-1 promoter induced activity in response to SSc and control conditioned media.

Sub-confluent mink lung epithelial cells containing the PAI-1 promoter linked to firefly luciferase were incubated in the presence TGF- β (0, 0.001, 0.01, 0.1, 1, 5, and 10 ng ml⁻¹) in the presence of IgG or 1D11 at 10 μ g ml⁻¹ (A), or conditioned media from control or SSc cell lines in the presence of IgG or neutralising TGF- β antibody, 1D11 (B). Luciferase activity assessed, and all values are normalised for basal expression of un-stimulated PAI-1 luciferase reporter activity (22 RLU \pm 5). Latent TGF- β levels represent total RLU of heat-activated medium minus RLU generated by the active form. All cell monolayers were assessed for necrosis. All samples were tested in triplicate and results presented represent the mean \pm S.E.M of CM from four cell lines.

4.4.5 Expression of Smad3, 4 and 7 in SSc fibroblasts

In Section 3.4.6 Smad3 and Smad4 were shown to mediate TGF- β 1 induction of CTGF in normal fibroblasts. The work already described in this chapter has shown SSc cells to be refractory to TGF- β 1 stimulation and the higher basal expression of CTGF to be independent of TGF- β . Thus the total protein levels of Smad3, Smad4, Smad7, and the nuclear levels of Smad3, were assessed by Western blot and protein levels semi-quantified by densitometry analysis (Figure 4.5). No difference in total protein levels of Smad4 (Control: 2 ± 0.25 SEM versus SSc: 1.8 ± 0.33 SEM) or the inhibitory Smad, Smad7 (Control: 2.2 ± 0.15 SEM versus SSc: 1.9 ± 0.23 SEM) were observed in SDF compared to control cells (Figure 4.5A). Detection of total Smad3 was variable; however no significant difference in the amount of protein was observed between control and SSc levels (Control: 1.1 ± 0.65 SEM versus SSc: 1.5 ± 0.93 SEM). Nuclear levels of Smad3 in the SDF also showed no difference compared to control (Control: 1.6 ± 0.29 SEM versus SSc: 1.7 ± 0.13 SEM) (Figure 4.5B).

4.4.6 Role of Smads in CTGF expression in SSc fibroblasts

Transgene activity of reporter constructs was assessed in order to investigate the role of SMAD-dependent signalling in the elevated level of CTGF in SSc. SDF exhibited a significant ($p < 0.05$) increase in full length CTGF800 promoter activity compared to control cells (Figure 4.6A). Co-transfection with Smad7 had no effect on the raised basal expression of CTGF in SDF (Figure 4.6A). Control and SDF transiently transfected with full-length CTGF800 promoter/SEAP reporter construct were compared to ones either containing a mutated SMAD or BCE-1 binding site. Mutation of the SMAD binding site had no significant effect on the basal level of promoter activity either in normal or SDF (Figure. 4.6B; $p > 0.05$). Thus, the high level of CTGF promoter activity observed in SDF was independent of the SMAD recognition sequence. In support of this observation no difference ($p > 0.05$) in luciferase levels was found between SSc or control cells transiently transfected with the CAGA reporter construct, containing eight multimerised copies of the SMAD3 consensus sequence, CAGA (Figure 4.6C).

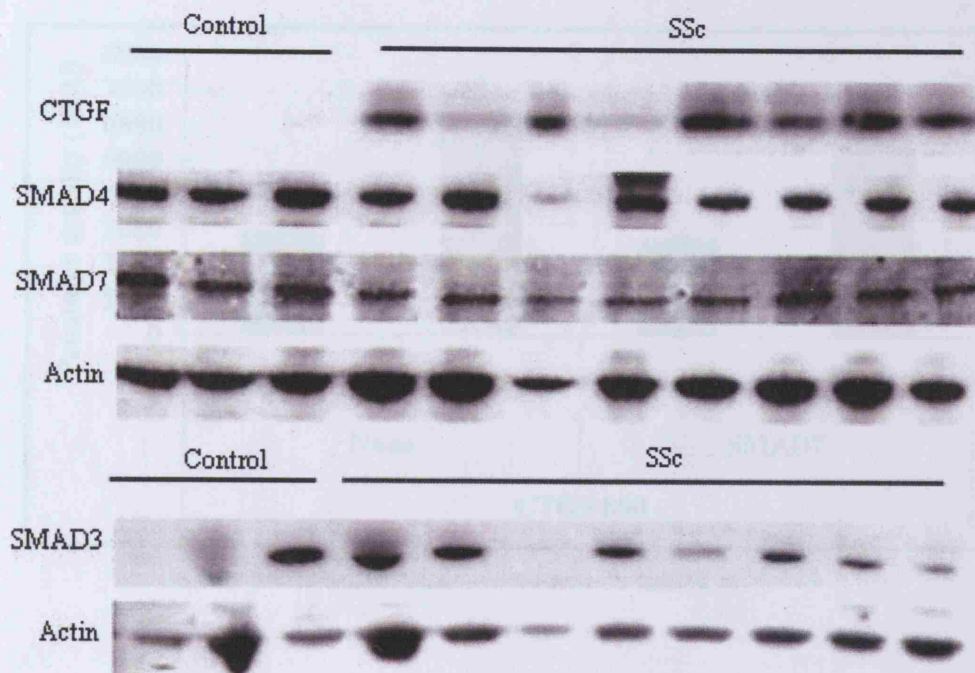
In order to determine whether the elevated level of CTGF promoter activity in SSc cells reflected elevated levels of basal promoter activity, a CTGF BCE-1 mutant promoter/SEAP reporter construct was transfected into normal and SDF. Mutating this element reduced CTGF promoter levels in both normal and SSc cells significantly

($p < 0.05$). Removing this element reduced CTGF promoter levels observed in SDF to those in normal fibroblasts, suggesting that this element is involved with the high level of CTGF expression observed in SSc (Figure 4.6B).

4.4.7 Identification of the minimal CTGF promoter region which conferred over expression in SSc fibroblasts

A series of CTGF promoter deletion constructs were transfected into normal and SDF in order to identify the regions of the CTGF promoter important for over-expression of the protein in SDF. All constructs exhibited 3 fold higher CTGF reporter activities in SDF compared to control (Figure 4.7), suggesting that the elevated level of CTGF promoter activity observed in SDF was controlled by the first 86 base pairs upstream of the transcription initiation start site. This region of the CTGF promoter does not contain the TGF- β 1 response element that is necessary for TGF- β to induce CTGF in control fibroblasts.

A



B

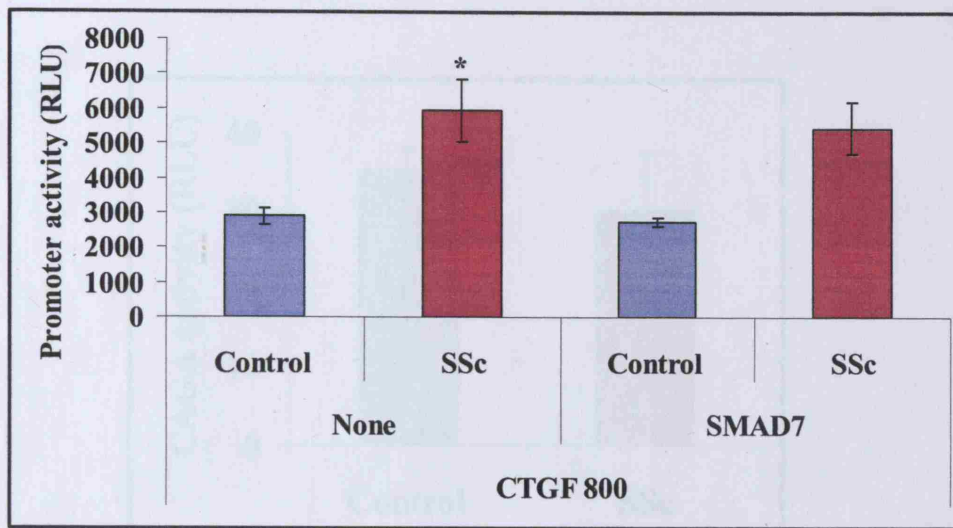


Figure 4.5

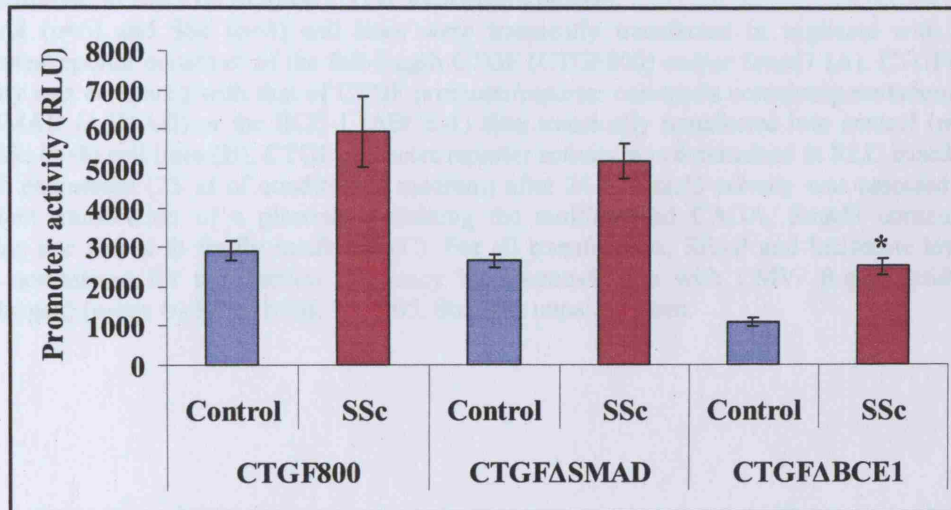
Protein expression of Smads 3, 4, and 7 in normal and SDF.

Western blots of total Smad3, Smad4 and Smad7 were performed on control and SDF monolayers and normalized to actin levels (A). Nuclear extracts from fibroblasts cultured from three control and three individuals with SSc with the highest CTGF expression were probed for Smad3 levels by Western blot (B). Protein expression levels were semi-quantified as described in Chapter 2, Section 2.6.

A



B



C

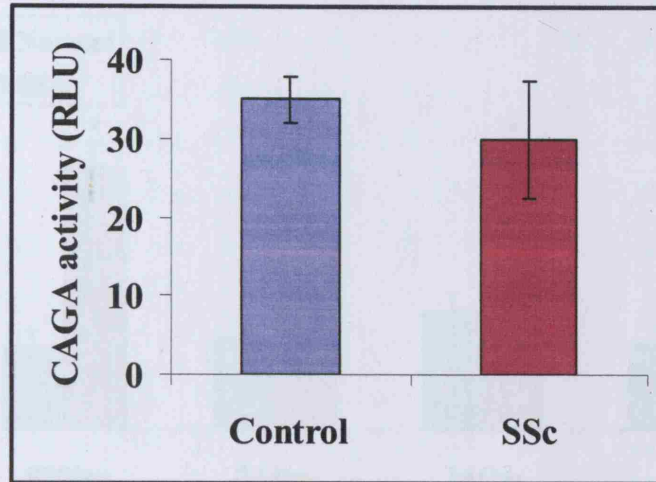


Figure 4.6

Contribution of SMADs to basal CTGF expression in SDF.

Control (n=6) and SSc (n=8) cell lines were transiently transfected in triplicate with the promoter/reporter construct of the full-length CTGF (CTGF800) and/or Smad7 (A). CTGF800 activity was compared with that of CTGF promoter/reporter constructs containing mutations in the SMAD (Δ SMAD) or the BCE-1 (Δ BCE-1) sites transiently transfected into control (n=6) and SSc (n=8) cell lines (B). CTGF promoter/reporter activity was determined in RLU based on SEAP expression (25 μ l of conditioned medium) after 24 h. Smad3 activity was assessed by transient transfection of a plasmid containing the multimerised CAGA, Smad3 consensus binding site linked to firefly luciferase (C). For all transfections, SEAP and luciferase levels were normalized for transfection efficiency by co-transfection with CMV- β -galactosidase (Invitrogen) (mean \pm SEM, n=6); *p<0.05, Students unpaired t-test.

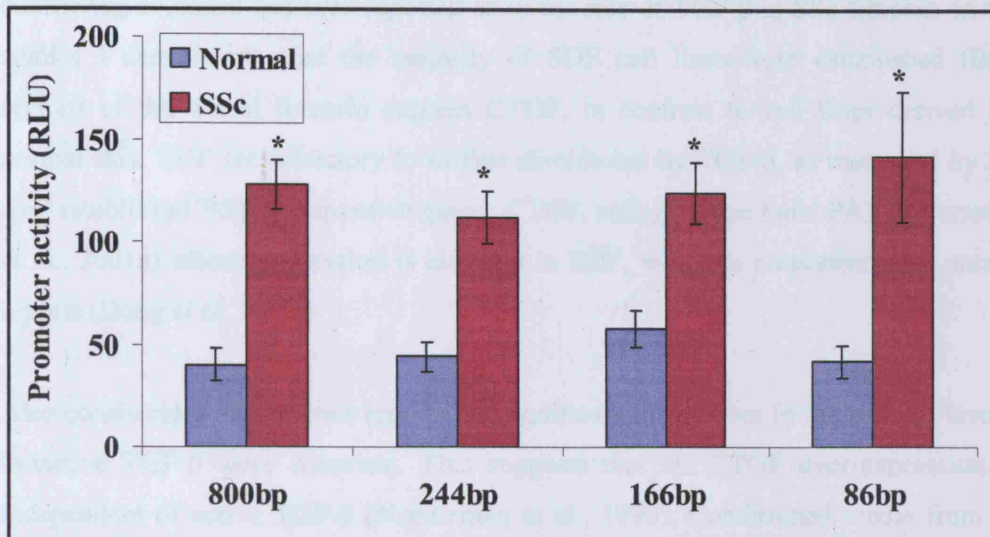


Figure 4.7

Deletion analysis of the CTGF promoter: sequences required for over-expression in SDF.

CTGF promoter/SEAP reporter constructs were transfected into dermal fibroblasts cultured from control (n=4) and SSc patients (n=6). Constructs were: the wild-type CTGF promoter (CTGF800, containing nucleotides -805 to +17), CTGF244 (containing nucleotides -244 to +17), and CTGF86 (containing nucleotides -86 to +17). Expression values (mean \pm S.D.), after adjusting for differences in transfection efficiencies among samples using a co-transfected vector (CMV- β -galactosidase (Clontech)), are shown. (*p<0.05)

4.5 Discussion.

This chapter has addressed the role of TGF- β and Smads in the over-expression of CTGF, in SDF from areas of established fibrosis. As discussed earlier in the chapter, conflicting evidence has been reported as to the role of TGF- β in SSc fibrosis. In these studies I demonstrate that the majority of SDF cell lines from established fibrotic regions of the dorsal forearm express CTGF, in contrast to cell lines derived from normal skin. SDF are refractory to further stimulation by TGF- β , as measured by three well established TGF- β responsive genes; CTGF, collagen type I and PAI-1 (Verrecchia et al., 2001a) whose expression is elevated in SDF, which is consistent with previous reports (Dong et al., 2002).

Also consistent with previous reports, no significant differences in the *in vitro* levels of bioactive TGF- β were observed. This suggests that the CTGF over-expression was independent of active TGF- β (Needleman et al., 1990). Conditioned media from both control and SDF induce PAI-1 activity in the presence of TGF- β neutralizing antibody suggesting factors other than TGF- β may activate the PAI-1 promoter. Only 70% of serum induction of PAI-1 mRNA and protein has been demonstrated to be attributable to TGF- β effects (Boehm et al., 1999). This implies that the induction of a proportion of the PAI-1 activity induced by fibroblast conditioned media is independent of TGF- β . Interestingly this inductive activity in conditioned media was lost by heat activation from control but not SDF, demonstrating the factor/s secreted by normal and SDF that activate the PAI-1 promoter in mink lung epithelial cells may differ either in their sensitivity to heat or are different from each other.

Neutralisation antibodies to TGF- β failed to inhibit the excessive expression of CTGF and collagen protein by SDF. Furthermore, this supports the previous observations that SDF produce similar amounts of active TGF- β to that of normal fibroblasts, as measured using mink lung epithelial cells. Interestingly, SDF produced significantly higher levels of latent TGF- β (Figure 4.4). Recent reports have shown thrombospondin-1, a converter of latent TGF- β to the active form, to be over-expressed in cultured SDF (Mimura et al., 2005). The lack of inhibition in this study of CTGF by the TGF- β neutralizing antibody cannot preclude either local activation of latent TGF- β at the cell surface or TGF- β levels exceeding the neutralising capacity of the antibody at the dose used. However, this latter notion is less plausible due to the lack of difference between the secreted levels of bio-active TGF- β by control and SSc cells found in the studies

presented in this chapter (Figure 4.5B). In contrast to earlier publications, TGF- β type I (ALK5) and type II receptors have recently been reported to be elevated on SDF. In addition, increased binding of TGF- β to the cell surface of SDF was observed (Yamane et al., 2002; Kawakami et al., 1998), whereas Varga's group have reported TGF- β ligand independent activation of Smads (Mori et al., 2003). The collective effects of over-expression of TGF- β receptors, ligand-independent activation of downstream signalling, and the increased conversion of TGF- β from latent to active in SSc remains unclear. Understanding the contribution of each variable has been hindered by having not been addressed in one complete study, and indeed each one may reflect different aspects and stages of the disease progression. However, the recent identification of a TGF- β receptor, ALK5, kinase inhibitor (Bonniaud et al., 2005) which is able to block fibrosis in the bleomycin-induced murine lung fibrosis model, would help in future studies to clarify the role played at the level of ligand receptor interactions in the disease phenotype. This would also address the specific role of the TGF- β signalling pathway in the regulation of CTGF in cells derived from established areas of fibrosis.

I have shown in Chapter 3 that Smad3 is required for the induction of CTGF by TGF- β in normal cells. Given the lack of effect of TGF- β neutralising antibodies on CTGF expression by SDF, the state of the Smad signalling pathway was assessed. Investigation of the Smad signalling pathway found no difference in either the expression of the co-Smad, Smad4, or the inhibitory Smad, Smad7. This is consistent with subsequent work by Mori *et al.* (Mori et al., 2003). In contrast, two reports have found diametrically opposed expression of Smad7 in SDF (Asano et al., 2004b; Dong et al., 2002). This is consistent with the observations presented here in that over-expression of Smad7 did not affect elevated CTGF promoter activity. Interestingly both groups found that transient over-expression of Smad7 did not affect either the elevated activity of the collagen 1 α 2 promoter (Asano et al., 2004b), or the higher PAI-1 basal transcription levels (Dong et al., 2002) in SDF, suggesting the elevated expression of these TGF- β responsive genes is independent of Smad activation.

The lack of consensus on the expression of Smad7 in SSc also extends to that of Smad3. In these investigations no changes in total or nuclear levels of Smad3 were observed (Figure 4.5B). In addition, promoter activity of a Smad3/Smad4 specific construct was not significantly different between control and SSc cells (Figure 4.6C), consistent with recent published reports (Asano et al., 2004b). Conversely, other groups found elevated

expression and nuclear localization of Smad3 (Dong et al., 2002; Mori et al., 2003). Differences in the anatomical origin of the fibroblasts, or the stage of the disease that they were obtained (i.e. inflammatory versus established), could explain this apparent discrepancy (Helman et al., 2005; Dong et al., 2002). In these studies I wished to address the mechanism underlying the over-expression of CTGF in established fibrotic areas. Therefore SDF were obtained from the affected areas (dorsal forearm) of patients with established fibrosis, whereas studies by Dong *et al.* obtained fibroblasts from unaffected areas -an area above the elbow considered to have normal skin thickness as determined by clinical palpation (Dong et al., 2002). Taken together, these studies suggest a dysregulated response to TGF- β may reflect temporal changes in the cell, or the cell types present, such as myofibroblasts (Kirk et al., 1995), as the disease progresses from initiation to persistent fibrosis (Helman et al., 2005; Denton and Black, 2004). Clearly a better clinical definition of the stages of the disease will help in elucidating the role of TGF- β and the signalling pathways involved. The data presented here suggest CTGF to be regulated independently of Smad3, at least in fibroblasts from affected areas of established fibrosis. Studies by Shi-wen *et al.* and others have shown CTGF to be expressed, albeit at lower levels, in fibroblasts derived from uninvolved areas (Shi-wen et al., 2000; Bonniaud et al., 2003). Future studies to determine the mechanisms controlling CTGF over expression in this situation, may clarify the role of Smads in the progression of fibrosis in SSc.

CTGF promoter/reporter constructs, lacking the TGF- β /Smad3 site in SDF, exhibited similar levels of elevated promoter activities to that of the wild type promoter (Figure 4.6B). Deletion of the BCE-1 element resulted in a proportional decrease in promoter activities, in normal and SDF, and is consistent with this element being involved in regulating basal expression. In order to define the minimal element of the CTGF promoter that confers the raised promoter/reporter activity, SDF were transfected transiently with a CTGF promoter deletion series (Figure 4.7). This identified an element within the first 86 bp of the minimal promoter which was sufficient for the elevated expression of CTGF in SDF. Promoter scanning of this region for DNA binding motifs identified two putative Sp1 binding sites, the investigation of which will be discussed in the following chapter.

4.6 Summary

In summation, the studies presented in this chapter have shown SDF are refractory to TGF- β 1 stimulation as determined by CTGF, collagen type I and PAI-1 expression. Furthermore, elevated expression of these same genes by SDF is independent of autocrine expression of active TGF- β . Consistent with increased CTGF expression being independent of TGF- β , CTGF promoter activity is independent of the TGF- β /Smad binding element and rather, dependent on elements contained within the first 86 bp of the CTGF promoter.

Chapter 5

Constitutive expression of CTGF in SSc fibroblasts is dependent on a Sp-binding site

5.1 Introduction

The results from Chapter 3 demonstrated TGF- β induction of CTGF in normal fibroblasts is Smad3 dependent. In the previous chapter neutralization studies and investigation of the Smad signalling pathway in dermal fibroblasts indicate over-expression of CTGF in SDF is independent of TGF- β /Smad activation. To determine the molecular mechanism underlying CTGF over-expression, SDF were transiently transfected with a series of deletion constructs and a region of the CTGF promoter necessary for the elevated promoter activity was identified. This region between -86/+17 was sufficient to confer higher basal expression of CTGF in SDF cells. In silico analysis of the promoter identified two putative Sp-binding sites (Figure 5.1), and the role of these sites will be explored in the studies presented within this chapter. In the following I shall review the current understanding of the Sp1-like family, focusing specifically on Sp1 and Sp3.

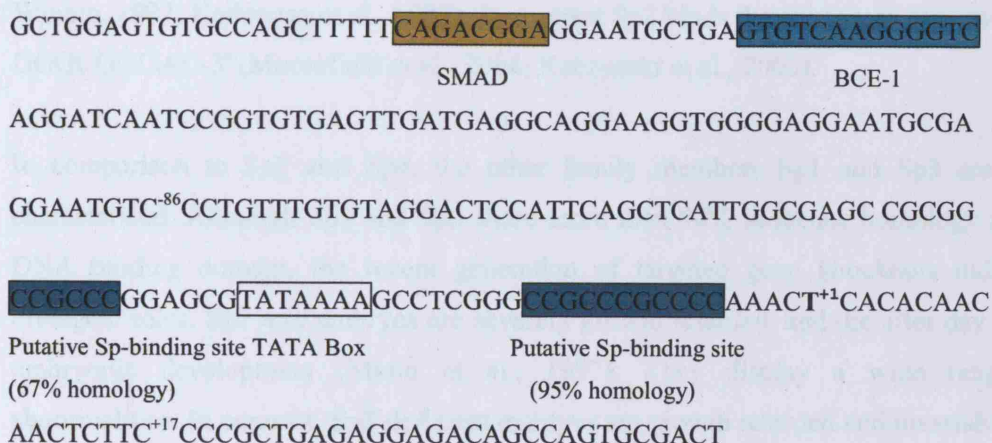


Figure 5.1

Sequence of the proximal upstream region of the CTGF promoter.

The previously characterised Smad3 binding site and BCE-1 element and two putative Sp-binding sites spanning the TATAA box are as indicated. ⁺¹ denotes transcriptional start site; ⁺¹⁷ denotes 3' end of the cloned genomic sequence of CTGF.

5.1.1 The Sp1 family of transcription factors

Sp1 was amongst the first eukaryotic transcription factors to be identified (Li et al., 2004; Kaczynski et al., 2003). Purified from fractionated HeLa cell extracts, Sp1 was cloned by Tijan *et al.* by virtue of the ability to bind the GC-rich DNA motif of the SV40 promoter (Dyner and Tijan, 1983b; Kadonaga et al., 1987; Dyner and Tijan, 1983a). The founding member of a 21 protein family, Sp1, like all Sp1-like/Kruppel-like factors (KLF) family members, contains three zinc fingers which form the DNA-binding domain of these transcription factors (Kadonaga et al., 1987; Kaczynski et al., 2003; Bieker, 2001; Kingsley and Winoto, 1992; Matsumoto et al., 1998). The Sp1-like/KLF family of transcription factors is divided into three general sub-groups. Sub-group 1 consists of the proteins most highly related to Sp1. The other Sp1-like/KLF proteins are divided into two additional groups comprising the kruppel-like factors and include the TIEG (TGF- β inducible early gene) genes, which have been implicated in TGF- β and CTGF signalling (Wahab et al., 2005b; Cook et al., 1998; Kaczynski et al., 2003). The Sp1 family members have a similar modular structure, and whereas Sp1 and Sp3 are ubiquitously expressed, Sp4 is predominantly expressed in the brain. To date little is known about Sp2 expression (Figure 5.2). Sp1, Sp3 and Sp4 are more closely related to each other than to Sp2. Consistent with this Sp1, Sp3 and Sp4 all bind the classical GC rich DNA region and the related GT/CACC box with identical affinity (Kingsley and Winoto, 1992; Kadonaga et al., 1987). In contrast Sp2 binds the consensus sequence: 5'-GGGCGGGAC-3' (Moorefield et al., 2004; Kaczynski et al., 2003).

In comparison to Sp2 and Sp4, the other family members Sp1 and Sp3 are well characterised. Although Sp1 and Sp3 share more than 90% sequence homology in the DNA binding domain, the recent generation of targeted gene knockouts indicates divergent roles. Sp1 *null* embryos are severely growth retarded, and die after day 10 of embryonic development (Marin et al., 1997). They display a wide range of abnormalities. In contrast, Sp3-deficient embryos are growth retarded and invariably die at birth of respiratory failure and show a pronounced defect in late tooth formation (Bouwman et al., 2000). Given that over 4000 publications have implicated Sp1 and Sp3 in gene regulation, the survival of the *null* embryos post implantation suggests a degree of redundancy in these transcription factors exists, at least during early embryonic development.

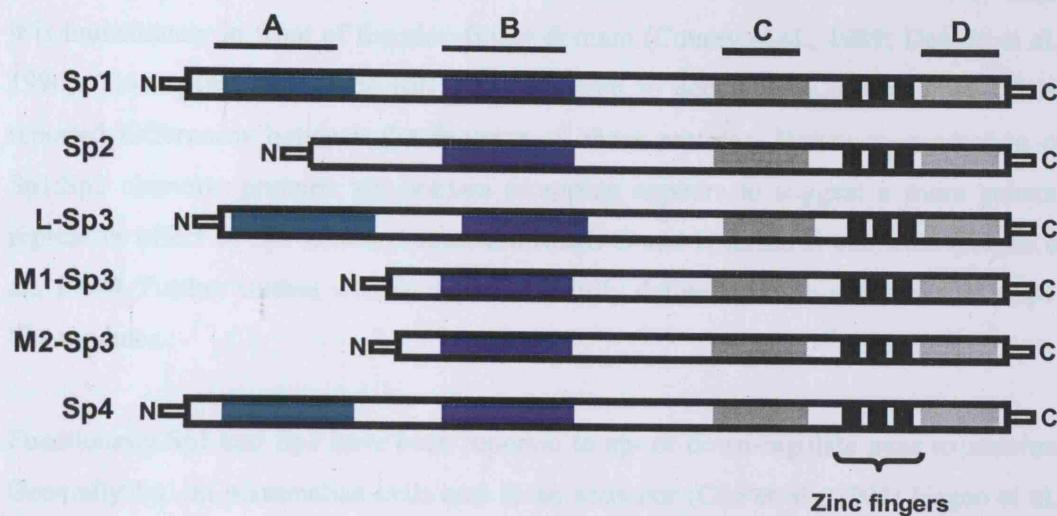


Figure 5.2

Structure of the Sp1 family members.

Schematic representation of the functional domains of Sp1, with a comparison of Sp1 with the related family members, including the 3 forms of Sp3. Long form (L-Sp3) and two short forms (M1, M2-Sp3). The black bars marked with A, B, C and D denotes Sp1 functional domains. The 3 zinc finger domains are the G-C box DNA-binding sites. Figure modified from Li *et al*; Liania *et al.* (Lania et al., 1997; Li et al., 2004).

5.1.2 Structural analysis of Sp1 family

Structurally, in addition to the three zinc fingers that confer DNA binding ability on Sp1 transcription factors, a series of four domains are required for transcriptional activity (See Figure 5.2). Two of these domains, A and B, correspond to glutamine-rich regions that are able to interact with the basic transcriptional machinery by binding to the TATA-binding protein (TBP) part of the TFIID complex, and this interaction is required for transcriptional synergy to occur (Gill et al., 1994; Gupta et al., 1996; Emili et al., 1994). Domain C contains a region of highly charged amino acids. At the carboxyl-terminal region lie the Cys2His2 zinc fingers, characteristic of all the Sp1-like/ KLF family (Lania et al., 1997). Domain D at the carboxyl terminus is required for the synergistic activation in conjunction with domains A and B and is thought to be involved in the formation of homomeric complexes (Courey et al., 1989; Mastrangelo et al., 1991). In addition Sp1 but not Sp3 forms higher order homomeric complexes through its D domain and is synergistically able to interact with multiple DNA binding sites (Mastrangelo et al., 1991). Though generally structurally similar, these ubiquitously expressed Sp1-like proteins have distinct functional differences.

Interestingly, whereas the inhibitory domain of Sp1 is located at the N-terminus, in Sp3 it is immediately in front of the zinc-finger domain (Courey et al., 1989; Dennig et al., 1996). This spatial difference has been proposed to account for the majority of the reported differences between the function of these proteins. However, generation of Sp1:Sp3 chimeric proteins via domain swapping appears to suggest a more general repressive effect of Sp3 action mediated through C and N terminal elements (Hagen et al., 1994). Further studies will be required to fully define the differences of these Sp1-like modules.

Functionally Sp1 and Sp3 have been reported to up- or down-regulate gene expression. Generally Sp1 in mammalian cells acts as an activator (Cao et al., 2003; Hagen et al., 1994; Kumar and Butler, 1998; Li et al., 2004; Motojima et al., 2000), while Sp3 appears to be able to function to activate and repress gene transcription (Cao et al., 2003; Hagen et al., 1994; Li et al., 2004; Choi et al., 2005). In addition, the formation of homomeric complexes by Sp1 synergistically activates promoters with two or more adjacent Sp-binding sites and allows the interaction of sites separated by several kilo base pairs or more (Yu et al., 2003a; Huang et al., 2005; Pardali et al., 2000; Turner and Crossley, 1999). Thus Sp1 and Sp3 effects may be controlled by the context and number of Sp-binding sites within a given promoter. Interestingly whereas Sp1 is expressed as a single 105 kDa protein the Sp3 protein has three isoforms; the full length L-Sp3 (115 kDa) and two shorter forms, M1-Sp3 (80 kDa) and M2-Sp3 (78 kDa), generated through differential translation initiation (Kennett et al., 1997). Recent reports suggest these shorter forms (Figure 5.2) that lack domain A but retain the inhibitory domain are responsible for the repressive actions of Sp3 (Choi et al., 2005). The ability of Sp3 in mammalian cells to activate or repress gene transcription may well reflect the ratio of long and medium proteins present conferring a further level of control on this protein.

Sp1 and Sp3 are post-transcriptionally modified via glycosoylation and phosphorylation (Jackson et al., 1990; Merchant et al., 1999; Han and Kudlow, 1997). The functional effects of this will be addressed in detail in Chapter 6. Sp1 and Sp3 regulate gene expression by co-operating with other proteins- including transcriptional activators and repressors. Several protein binding sites have been identified in Sp1, both within the A and B domains as well as the C-terminal and DNA-binding (Zinc finger) domain (Gartel and Shchors, 2003; Gill et al., 1994; Lania et al., 1997). The ability of Sp1 to form higher order homomeric complexes allows for the multiple binding of interacting

proteins and may go some way to explaining the role of Sp1 as a transactivator (Hagen et al., 1994). Sp1 and Sp3 interact with transcription-associated proteins (Gill et al., 1994), sequence specific DNA binding proteins such as Smads (Gill et al., 1994; Chamboredon and Castellazzi, 2005; Inagaki et al., 2001) and chromatin remodeling factors (e.g. p300, histone deacetylase 1 (HDAC1)) (Suzuki et al., 2000; Doetzlhofer et al., 1999). The recruitment by Sp1-like factors, such as Sp1, of these proteins to form complexes on the gene promoter can cause region-specific changes in histone acetylation and polymerase II recruitment within promoters. The recruitment and stabilisation of transcription factors to DNA by Sp1 promotes gene expression (Docagne et al., 2004; Motojima et al., 2000; Chadjichristos et al., 2003; Chamboredon and Castellazzi, 2005; Dennig et al., 1996). In turn repressive interactions with other transcription factors may interfere with Sp1 DNA binding and recruitment leading to repression of Sp1 mediated transcription, for example c-myc inhibits Sp1 activity (Gartel and Shchors, 2003).

Thus the Sp1-like action of Sp1 and Sp3 is controlled at multiple levels, through the dynamic interaction with kinases and phosphatases, the expression of two shorter Sp3 isoforms, and the ability of Sp1 but not Sp3 to form homomeric complexes that span Sp-binding sites. In addition, the ratio of expression of Sp1 and Sp3, the recruitment of accessory proteins, and the proteins that may interact with them leads to a complex mechanism of control of gene transcription mediated by these ubiquitously expressed proteins.

5.1.3 Sp1 and Sp3 in collagen regulation

Several studies have shown the involvement of Sp1 and the importance of Sp1 binding to *cis*-elements encoding the $\alpha 1$ and $\alpha 2$ chains of type I collagen (Ghayor et al., 2001; Artlett et al., 1998; Ihn et al., 1996; Pogulis and Freytag, 1993; Greenwel et al., 1997; Tamaki et al., 1995). Though an early study by Nehls *et al.* suggested a negative transcription role for Sp1 subsequent studies by the same group using the Sp1-deficient Schneider L2 cells, discounted Sp1 inhibitory effects on collagen (Nehls et al., 1991; Nehls et al., 1992). Sp3, as well as acting as a repressor, is also considered to transactivate promoters, much of these studies being performed in Schneider cells, absent for all Sp1 family members. Like Sp1, Sp3 has been shown to up-regulate COL1A2 in these cells (Ihn and Trojanowska, 1997). However, recent transfection studies in mammalian cells have shown Sp3 to repress the trans-activation of the

COL2A1 gene by Sp1 (Ghayor et al., 2001; Majello et al., 1997), presumably through the competition of binding sites with Sp1. Sp3 is now proposed to act as a bi-functional transcriptional regulator and this function is predominantly dependent on the context of the Sp DNA-binding sites. In the context of collagen regulation its role may be cell specific (Inagaki et al., 2001). Given the growing number of papers demonstrating the ability of Sp1 to regulate collagen, the role of Sp1 in fibrosis has come under closer scrutiny. In Ito cells, the primary cell type responsible for elevated collagen expression in liver fibrosis, Sp1 binding has been demonstrated to be elevated (Rippe et al., 1995). Indeed, work published on the commencement of these studies has shown nuclear extracts harvested from SDF possessed increased binding of Sp1 to the COL1A1 promoter (Ihn and Tamaki, 2000). There is therefore increasing evidence to show that increased binding and transcriptional activation to Sp transcription factors may play a significant role in fibrotic conditions and thus targeting of these may lend itself to novel anti-fibrotic therapies.

5.1.4 Inhibition of Sp1

Mithramycin (trade name Pilcamycin) is an aureolic acid anti-neoplastic antibiotic that is used for treating cancer-related hypercalcemia (Zojer et al., 1999). Previous work has revealed that it inhibits bone resorption *in vitro*, possibly by interfering with bone cell lysosomal enzymes (Kiang, 1978). It also prevents the binding of the Sp1 transcription factor to its cognate site in DNA, selectively inhibiting RNA synthesis from genes with GC-rich promoter sequences (Blume et al., 1991). Mithramycin has thus been used to examine the effects of Sp1 inhibition on TGF- β 1 induction of the COL1A2 gene (Poncelet and Schnaper, 2001). Indeed, a recent study showed targeted interference of Sp-binding sites using mithramycin was able to inhibit the elevated expression of collagen by scleroderma cells (Ihn and Tamaki, 2000). Work by Mauviels group, examining the effects of targeting Sp1 levels through anti-sense expression vector and cDNA microarrays analysis has demonstrated that the reduction of Sp1-induced extracellular matrix gene mRNA steady-state levels results from transcriptional repression of extracellular matrix genes including COL1A1, COL1A2, COL3A1, COL5A2 and COL7A1 (Verrecchia et al., 2001b). Thus there is much evidence both to suggest Sp1 dysregulation has a functional role to play in fibrosis and may represent an attractive target for therapy. In the following studies I will investigate the functional relevance of the putative Sp-binding sites identified in the CTGF proximal promoter and contribution to CTGF over-expression in SSc.

5.2 Aims

The following studies will explore the functional relevance of the Sp-binding sites in the CTGF promoter in normal and diseased cells. The aims of the current chapter were:

- 1- To identify the transcriptional binding sites and transcription factors binding the 80bp upstream region of CTGF in normal fibroblasts.
- 2- To determine the functional relationship of these sites with over-expression of CTGF in SDF.

5.3 Materials and Methods

5.3.1 Cell culture

Control and SSc fibroblast cell lines, all previously characterised as expressing high levels of CTGF (Chapter 3) were used in the course of the studies presented within this chapter. All primary dermal fibroblast cell lines were maintained in FGM media as described in Section 2.2.1. For all experiments cells were used between passage 2 and 5 and cells were cultured until fully confluent and assayed after 18h in low serum.

5.3.2 Protein expression

All antibodies were as described in Section 2.4.4, and all protein extraction and Western blotting methodologies performed as described in Section 2.4.3, unless stated otherwise.

5.3.3 Mithramycin

For studies involving mithramycin (Sigma Aldrich), 150 nM mithramycin (Sigma) or solvent (Methanol) alone was added 6 h before harvesting.

5.3.4 DNA constructs

The luciferase reporter construct pGAG6, a kind gift of Professor Andrew Kudlow (UAB, USA), contains six multimerised consensus Sp1-binding sites (Kumar and Butler, 1998). The Sp1 expression vector was a generous gift from Dr Dimitris Kardassis (University of Crete, Greece) and the Sp3 vector (Birnbaum et al., 1995) from Professor Suske (University of Marburg, Germany).

5.3.5 EMSA oligonucleotide

Consensus double stranded Sp1 and Ap2 oligonucleotides (Promega). CTGF double stranded probes (Table 5.1) were generated as previously described in Section 2.5.2.

5.3.6 Sp1 DNA protein ELISA

Levels of Sp1 transcription factor DNA interactions were determined using an ELISA based approach (Clontech) and were performed as per the manufacturer's instructions. Briefly, nuclear extracts from control and SSc cell lines grown to confluence in 10cm² tissue culture petri dishes were isolated as previously described (Section 2.5.2). In general, a yield of 50 to 120 µg protein per dish of nuclear extract was isolated.

To a 96 well flat bottom plate, either 10 µg of sample or 5 µg of control HeLa nuclear extract in a total volume of 10 µl was added to 40 µl of binding buffer containing the biotinylated Sp1 probe. Samples were incubated at room temperature for 30 min with mild agitation (100 rpm) on a rocking platform and the protein/DNA complex allowed to form. The streptavidin-coated assay plate was washed 3x with wash buffer (200 µl per well), and 45 µl of sample transferred from each well to the streptavidin-coated assay plate. Samples were incubated for 1h at room temperature with mild agitation to allow the biotinylated Sp1 probe/Protein complex to bind to the streptavidin-coated assay plate. Each well was washed as described, 100 µl of Sp1 primary antibody (1:200 dilution) added to each well, and incubated for a further 1h at room temperature. Primary antibody was removed and each well washed. 100 µl of horseradish peroxidase-conjugated secondary antibody (1:1000) was added per well and the assay plate incubated at room temperature for 1h with mild agitation. Following a further 3 washes, 100 µl of pre-warmed substrate solution containing TMB was added and the assay plate incubated in the dark for 10 min at room temperature, after which 100 µl stop solution was added and the absorbance at 450 nm determined in a spectrophotometer. All samples were performed in duplicate.

Name	Oligonucleotide (5'-3')
CTGF-Sp1-WT (sense)	GAGCCGCGGCCCGCCCGGAGCGTATAAAAGCCTCGGGCCGCC CGCCCCAACTCACACAACAACCTCTCC
CTGF-Sp1-WT (anti-sense)	GGAAGAGTTGTTGTGTGAGTTTGGGGCGGGCGGCCCGAGGCTT TTATACGCTCCGGGCGGCCGCGGCTC
5'-Sp1 CTGF (sense)	GAG CCG CGG AAG CTT GGA GCG TAT AAA AGC CTC GGG CCG CCC GCC CCA AAC TCA CAC AAC AAC TCT TCC
5'-Sp1 CTGF (anti-sense)	GGA AGA GTT GTT GTG TGA GTT TGG GGC GGG CGG CCC GAG GCT TTT ATA CGC TCC AAG CTT CCG CGG CTC
3'-Sp1 CTGF (sense)	GAG CCG CGG CCG CCC GGA GCG TAT AAA AGC CTC GGG CCA AGC TTC CCA AAC TCA CAC AAC AAC TCT TCC
3'-Sp1 CTGF (anti-sense)	GGA AGA GTT GTT GTG TGA GTT TGG GAA GCT TGG CCC GAG GCT TTT ATA CGC TCC GGG CGG CCG CGG CTC

Table 5.1

CTGF oligonucleotides for EMSA.

CTGF oligonucleotides of the CTGF promoter (WT) Sp-binding sites are indicated in bold and mutations in 5' and 3' putative Sp-binding sites. The 5'Sp1 and 3'Sp1 competitors are identical to the probe (WT), other than that they contain a mutation to a HindIII (AAGCTT) site in the appropriate putative binding site.

5.4 Results

5.4.1 Functional identification of an Sp-binding site in the CTGF proximal promoter.

In silico analysis of the CTGF promoter between -86 and +17 for DNA binding motifs (Figure 5.1) identified two putative Sp-binding sites flanking the TATA box (See Figure 5.3A). To determine if this region contained a functional Sp-binding site an EMSA analysis was performed. A strong complex was formed in the presence of HeLa nuclear extract (Figure 5.3 B; lane 2). Incubation with 100 fold molar excess of unlabelled cold consensus Sp but not Ap2 or Smad oligonucleotides (Figure 5.3 B; lanes 3-5 respectively) completely abolished the ability of this complex to interact with the CTGF promoter. Incubation with unlabelled cold competitor of the CTGF promoter -86 and +17 (WT) was able to completely abolish binding (Figure 5.3 B; lane 6). Whereas incubation with a 100 fold cold competitor containing a mutation in the putative 5' but not 3' Sp1 binding site, did not inhibit the ability of Sp1 binding to the radio-labeled CTGF promoter region (Figure 5.3 B; lane 7), a cold competitor with a mutation in the 3' but an intact 5' Sp1 binding site did inhibit protein binding to the radiolabelled CTGF promoter region. Confirming the 5' Sp-binding, but not the 3' Sp-binding, site was able to bind protein.

5.4.2 Sp1 and Sp3 transactivate the Sp-binding site reporter construct pGAG6

In order to confirm the efficacy of the Sp1 and Sp3 expression vectors both were transiently transfected into NIH-3T3 containing a luciferase reporter construct of 6 multimerised Sp-binding (CG-rich region) consensus sequences (Kumar and Butler, 1998). Co-transfection of the Sp1 over-expression vector significantly ($p < 0.05$) increased luciferase reporter gene expression of the promoter reporter construct containing 6 multimerised Sp-binding sites, pGAG6, 4 fold, whereas Sp3 induced a 2 fold increase (Figure 5.4). Co-transfection of both Sp1 and Sp3 induced a similar 2 fold induction to that of Sp3 alone but significantly ($p < 0.05$) lower than Sp1 alone. Transfection of Sp1 or Sp3 over-expression vectors had no significant effect on reporter activity on the background vector, pGAG.

GAGCCGCGGCCGCCGGAGCGTATAAAAGCCTCG
5' Sp1 TATA box
GGCCGCCGCCCCAACTCACACAACACTCTTC
3' Sp1

Competitor - - Sp1 Ap2 SMAD WT Δ3' Δ5' Sp1 Sp1

Sp →
binding

Free Probe

(A) Sequence of the CTGF promoter (WT). Putative Sp1 sites are underlined (5' Sp1 and 3' Sp1) and are defined relative to the TATA box (grey). (B) The sequence shown in (A) was synthesized as a double-stranded oligomer, radiolabeled, and used in a gel-shift assay with HeLa nuclear extracts (10ug/reaction) that contained Sp1 binding activity (Lane 2). Competitor oligonucleotides were used as indicated at 100-fold molar excess. Cold competitors comprising either the wild type (WT) sequence or a mutation in either the 5' (Δ 5'Sp1) or 3' (Δ 3'Sp1) Sp1 sites (Table 5.1) were used as indicated.

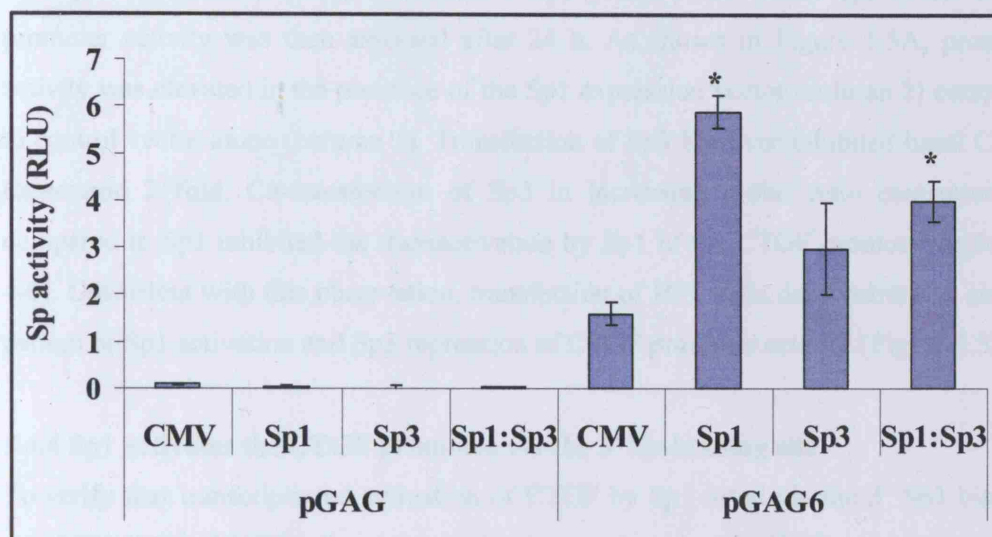


Figure 5.4

Effect of Sp1 and Sp3 expression vectors on the Sp1 reporter construct pGAG6 vectors.

The synthetic promoter pGAG6, containing 6 multiple consensus Sp-binding sites, or the vector alone were transfected into NIH 3T3 in the presence of expression vector alone (CMV); Sp1 alone, Sp3 alone, or an equal molar ratio of Sp1:Sp3. After 24 h incubation in serum-free media, luciferase activity was measured and normalized to transfection efficiency by β -galactosidase activity via co-transfection with CMV- β -galactosidase. Data shown is representative of 2 experiments with n=3 wells; mean \pm SEM. * p <0.05, Students unpaired t-test. There was no significant induction by Sp1 or Sp3 of the control vector, pGAG basic.

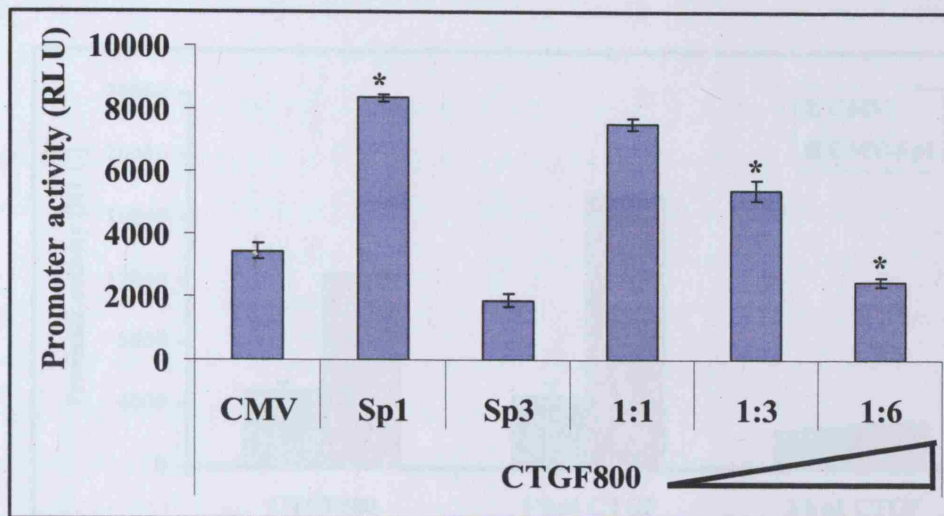
5.4.3 Regulation of CTGF by Sp1 and Sp3

Both NIH 3T3 and HFF cells were transiently co-transfected with the CTGF -804/+17 promoter/reporter construct, CTGF800, in combination with either the background expression vector CMV3.1 (CMV), Sp1, Sp3, or increasing molar ratios of Sp3 to Sp1 i.e. equal molar ratio (1:1); 3 fold more Sp3 (1:3); 6 fold more Sp3 (1:6). CTGF promoter activity was then assessed after 24 h. As shown in Figure 5.5A, promoter activity was elevated in the presence of the Sp1 expression vector (column 2) compared to control vector alone (column 1). Transfection of Sp3 however inhibited basal CTGF expression 2 fold. Co-transfection of Sp3 in increasing molar ratio concentrations compared to Sp1 inhibited the transactivation by Sp1 of the CTGF promoter (columns 4-6). Consistent with this observation, transfection of HFF cells demonstrated a similar pattern of Sp1 activation and Sp3 repression of CTGF promoter activity (Figure 5.5B).

5.4.4 Sp1 activates the CTGF promoter via the 3' Sp-binding site

To verify that transcriptional activation of CTGF by Sp1 acted via the 3' Sp1 binding site, NIH-3T3 and HFF cells were transiently transfected with CTGF promoter/reporter constructs containing mutations in either the 5' or 3' putative Sp-binding sites in the context of the -800 to +17 CTGF proximal promoter, and their activity compared to that of the unaltered wild type CTGF800 (WT CTGF) promoter. Mutation of the 3' Sp-binding site induced a 2 fold reduction in basal expression compared to CTGF800. Mutation of the 5' Sp-binding site mutant had no effect compared to CTGF800 promoter activity (Figure 5.6A). Co-transfection of Sp1 induced a 2.5 fold induction in WT CTGF activity confirming Sp1 was able to transactivate the CTGF promoter. Co-transfection of Sp1 induced CTGF promoter activity of the 5' but not the 3'Sp-binding site point mutants (Figure 5.6A). Results obtained while transfecting HFF cells were consistent with those observed with NIH 3T3 cells (Figure 5.6B).

A



B

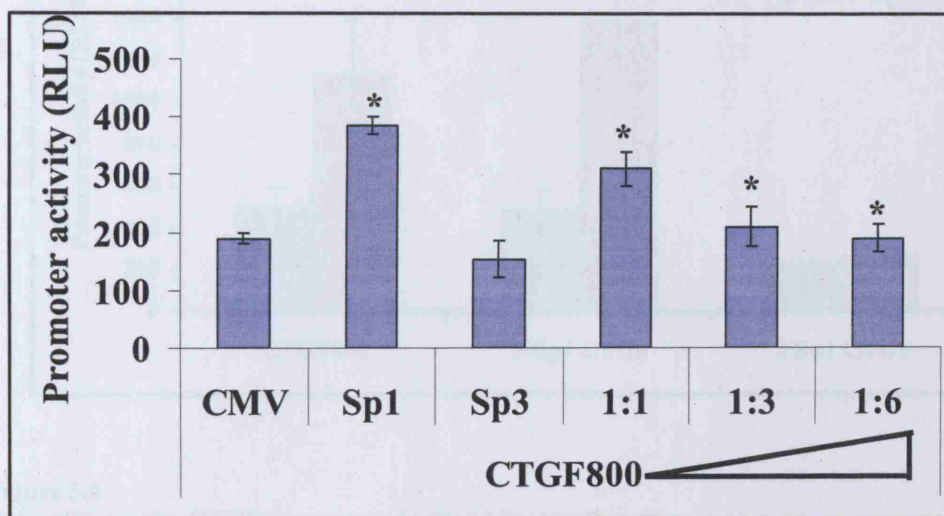
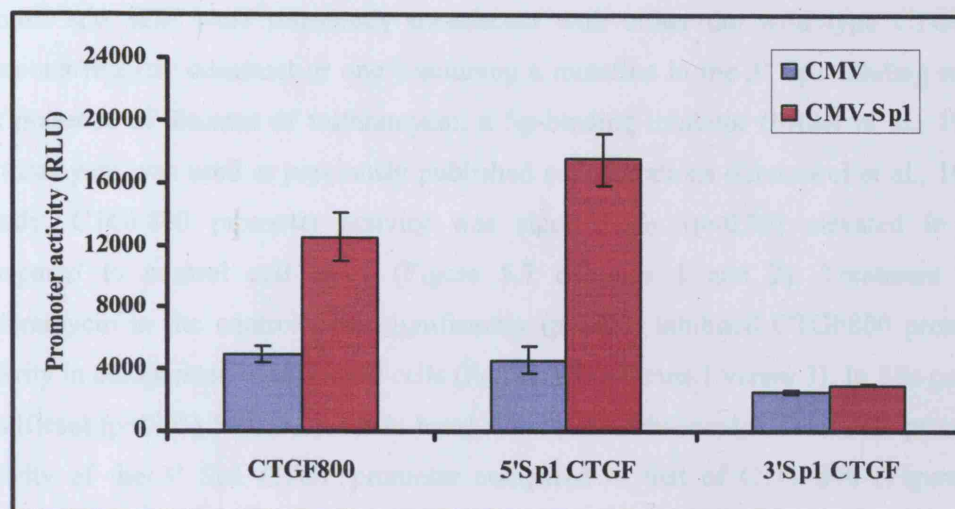
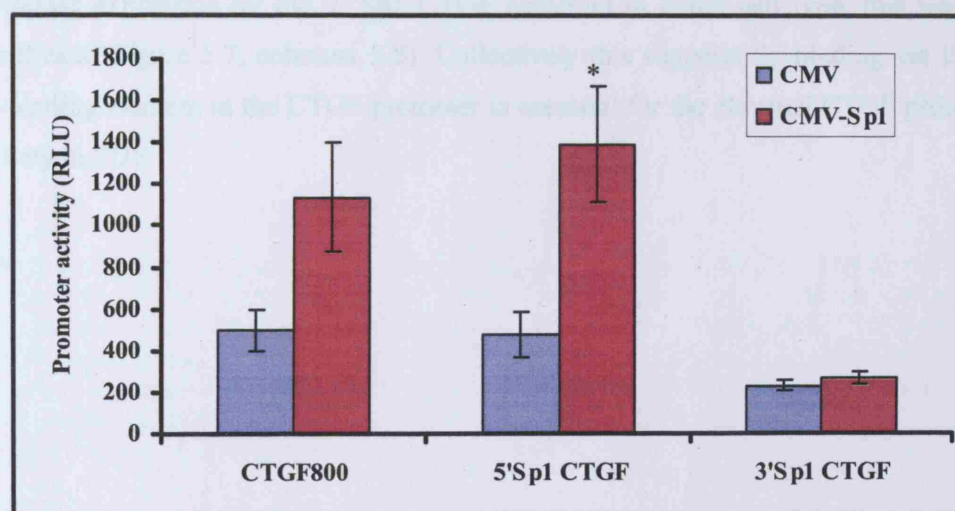


Figure 5.5

Effect of Sp1, Sp3 alone or in combination on CTGF promoter activity.

Near confluent NIH 3T3 (A) and HFF (B) cells were transiently transfected with the wild-type CTGF promoter/SEAP reporter construct (CTGF800, containing nucleotides -805 to +17) alongside 500 ng/well of either expression vector alone (lane 1); Sp1 alone (lane 2), Sp3 alone (lane 3) or Sp1:Sp3 at the indicated ratio (denoted by the black triangle). After 24 h incubation in serum-free media, SEAP activity was measured (25 μ l) and normalized for transfection efficiency by β -galactosidase activity via co-transfection with CMV- β -galactosidase. Data shown is representative of 2 separate experiments with n=6 wells; mean \pm SEM; *p<0.05, Students unpaired t-test. No significant induction by Sp1 and Sp3 of the control vector, SEAP basic, was noted (P>0.05).

A**B****Figure 5.6****Sp1 activates the CTGF promoter via the 3' Sp-binding site.**

The wild-type CTGF promoter/SEAP reporter construct (CTGF800) or constructs bearing mutations in either of the putative 5'Sp1 or 3'Sp1 sites in the CTGF promoter were transiently co-transfected alongside the empty expression vector or a vector encoding Sp1 into NIH 3T3 (A) and HFF (B) cells. After 24 h incubation in serum-free media, SEAP activity was measured (25 μ l) and normalized for transfection efficiency by β -galactosidase activity via co-transfection with CMV- β -galactosidase. Data shown is representative of 2 experiments with n=6 wells; mean \pm SEM, n=6; *p<0.05, Students unpaired t-test.

5.4.5 Sp-binding is required for elevated levels of CTGF promoter activity in SDF

To assess the contribution of Sp-binding to the elevated level of CTGF expression, control and SDF were transiently transfected with either the wild type CTGF800 promoter/reporter construct or one containing a mutation in the 3' Sp1 binding site in the presence or absence of mithramycin, a Sp-binding inhibitor (Miller et al., 1987). Mithramycin was used at previously published concentrations (Greenwel et al., 1997). Firstly, CTGF800 promoter activity was significantly ($p<0.05$) elevated in SSc compared to control cell types (Figure 5.7 columns 1 and 2). Treatment with mithramycin in the control cells significantly ($p<0.05$) inhibited CTGF800 promoter activity in comparison to untreated cells (Figure 5.7 columns 1 versus 3). In SSc cells, a significant ($p<0.05$) 50% decrease in basal activity was observed in the CTGF promoter activity of the 3' Sp1 CTGF promoter compared to that of CTGF800 (Figure 5.7 columns 2 versus column 6). Though treatment with mithramycin further reduced luciferase expression by the 3' Sp1 CTGF construct in either cell type, this was not significant (Figure 5.7, columns 5-8). Collectively this suggests Sp-binding via the 3' Sp-binding element in the CTGF promoter is essential for the elevated CTGF promoter activity in SDF.

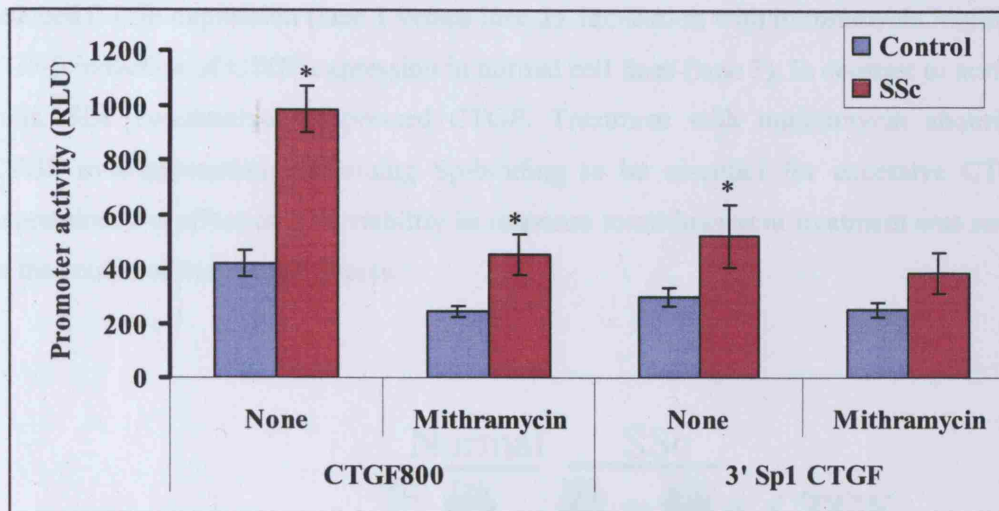


Figure 5.7

Effects of targeted disruption of the Sp-binding sites on CTGF promoter activity in SDF.

Control and SDF were transfected with either CTGF800 or 3' Sp1 CTGF promoter/SEAP reporter constructs in the presence or absence of mithramycin (150 nM). After 24 h incubation in low-serum media, SEAP activity was measured (25 μ l) and normalized for transfection efficiency by β -galactosidase activity via co-transfection with CMV- β -galactosidase. Data shown is representative of 3 control and 5 SSc cell lines transfected in triplicate, mean \pm SEM; * p <0.05, Students unpaired t-test.

5.4.6 Mithramycin represses CTGF over-expression in SSc dermal fibroblasts

To confirm the previous observation, two scleroderma lines were incubated in the presence of mithramycin and the effect on CTGF expression examined by Western blot. Normal cell lines did not express CTGF, however treatment with TGF- β potently induced CTGF expression (lane 1 versus lane 2). Incubation with mithramycin inhibited TGF- β induction of CTGF expression in normal cell lines (lane 3). In contrast to normal cells, SDF constitutively expressed CTGF. Treatment with mithramycin abolished CTGF over-expression confirming Sp-binding to be essential for excessive CTGF expression. No effect on cell viability in response to mithramycin treatment was noted in the course of these experiments.



Figure 5.8

Effect of mithramycin on CTGF expression in dermal fibroblasts.

After culturing cells to 85% confluence, media were changed, and the Sp1 inhibitor mithramycin (150 nM) was added for 24 h to normal dermal fibroblasts, in the presence or absence of TGF- β 1 (2.5 ng/ml), and to fibroblasts cultured from dermal lesions of patients with diffuse scleroderma. Cell layers were then lysed and equal amounts of protein (10 μ g) were subjected to SDS/PAGE and Western blot analysis with an anti-CTGF antibody. Addition of mithramycin blocks the basal CTGF expression in scleroderma fibroblasts. Blots were also probed with an anti-actin antibody to establish that lanes were equally loaded. Blot shown is representative of two separate experiments.

5.4.7 Sp1 and Sp3 levels in SSc fibroblasts

Results obtained by Western blotting can be seen in Figure 5.9 (A) of CTGF, Sp1 and the three Sp3 isoforms L-Sp3, M1-Sp3 and M2-Sp3, with protein levels normalised to actin protein levels and semi-quantified (Figure 5.9 B). CTGF was elevated in SSc compared to control cell lines, whereas there was no significant difference in protein levels of Sp1 or Sp3 isoforms (Figure 5.9).

5.4.8 Elevated Sp-binding and Sp1 in SSc dermal fibroblasts

Previous studies have shown Sp1 binding to DNA to be affected by post-translational modifications such as phosphorylation (Kumar and Butler, 1998; Merchant et al., 1999). To determine the binding ability of SSc nuclear extracts to Sp-binding sites, EMSA was performed using consensus Sp-DNA binding oligonucleotides. Both control and SSc nuclear extracts formed a complex with the radio-labeled DNA probe. However, nuclear extracts from SDF cell lines appeared to exhibit consistently stronger DNA protein complex formation compared to control (Figure 5.10A).

Because the inductive effects of Sp1 on CTGF promoter activity could be repressed by increasing the ratio of Sp3 : Sp1 (Figure 5.5) and total protein levels both of Sp1 and Sp3 are unaltered in SDF, a change in ratio in these transcription factors does not account for the elevated expression of CTGF (Figure 5.9). Using a DNA/Protein ELISA, the amount of functional Sp1 able to bind to Sp-binding oligonucleotides was quantified by spectrophotometer. SSc nuclear extract contained a significantly ($P < 0.05$) 2 fold elevated level of Sp1 binding compared to control (Figure 5.10B). Nuclear extracts from the epithelial cell line, Hela, were used as positive controls. Collectively this suggests that SSc fibroblast nuclear extract contains functionally higher levels of Sp1 able to bind to Sp-binding sites.

A



B

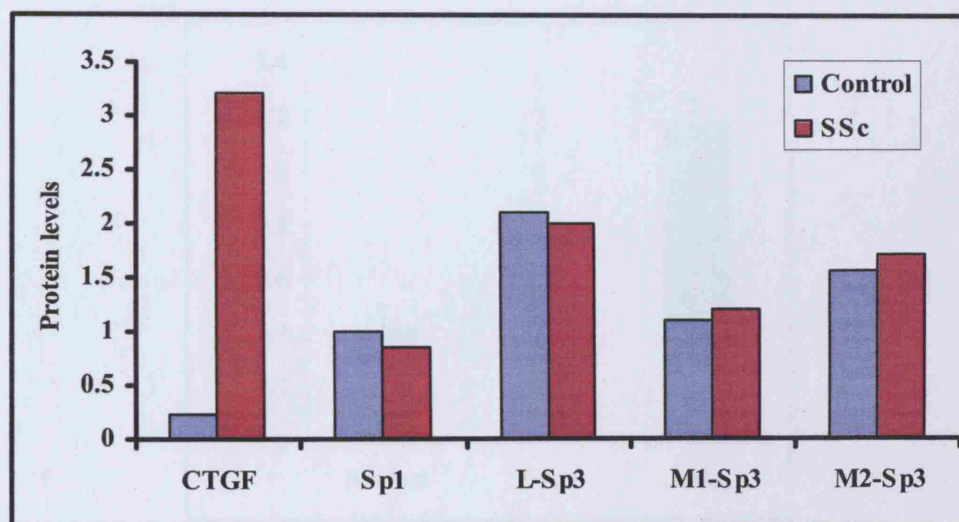


Figure 5.9

Expression of Sp1 and Sp3 in control and SDF.

Western blots were performed on mono-layers of 3 control and 8 confluent SDF lines characterised as high CTGF expressers for Sp1 and Sp3. Blots were also probed with an anti-actin antibody to establish equal protein loading (A). Mean relative protein expression (in arbitrary units) of CTGF, Sp1, L-Sp3, M1-Sp3 and M2-Sp3 normalised to actin expression were determined (B).

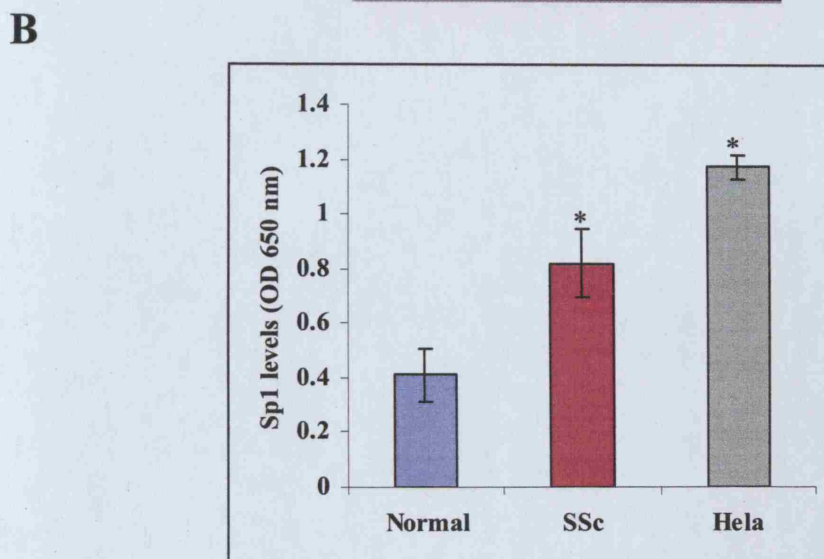
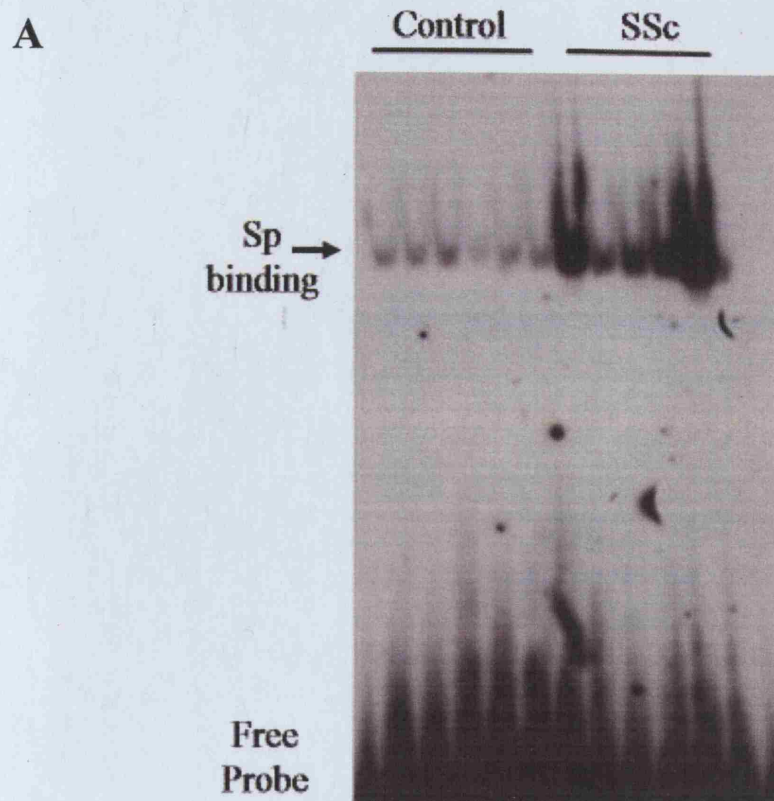


Figure 5.10

Elevated levels of Sp1 binding to DNA in nuclear extracts from fibroblasts cultured from dermal lesions of SSc patients.

Nuclear extracts were prepared from dermal fibroblasts cultured from six healthy subjects as well as five subjects with SSc. EMSA on nuclear extracts from control and SSc cells lines using consensus Sp1 oligonucleotides was performed (A). An ELISA based assay, which detects Sp1 binding activity to a double-stranded oligonucleotide bearing a consensus Sp-binding motif, was used to detect relative levels of Sp1 binding activities in nuclear extracts prepared from dermal fibroblasts cultured from four healthy subjects as well as five subjects with diffuse SSc (mean \pm S.D.). * $p < 0.05$, Students unpaired t-test (B).

5.5 Discussion

To address the mechanism by which the minimal region of the CTGF promoter, identified in Chapter 4, conferred the over-activity of the CTGF promoter/reporter construct in SSc cells, DNA-binding motifs within this region were identified. Two putative Sp-binding motifs, with 67% and 95% homology flanking the TATA box of CTGF were found (Figure 5.1). EMSA has identified a strong complex formed on this proximal region of the CTGF promoter that could be inhibited by either cold Sp1 consensus oligonucleotides or double stranded DNA oligonucleotides containing a mutation in the 5' (67% homology) Sp-binding site (Figure 5.3). Mutation of the 3' (95% homology) Sp-binding site did not inhibit the formation of this complex to the radiolabelled probe, suggesting that this protein complex is bound to the 5' Sp-binding site of the CTGF promoter.

Sp1 and Sp3 are two ubiquitously expressed members of the Sp1-like family of transcription factors that bind to Sp-binding sites (Kingsley and Winoto, 1992; Kadonaga et al., 1987). Over-expression of Sp1 induced CTGF promoter activity, whereas Sp3 reduced activity (Figure 5.5). An increasing molar ratio of Sp3:Sp1 inhibited activation of the CTGF promoter by Sp1 significantly (Figure 5.5) in both NIH/3T3 and HFF cells. This suggests that in the context of CTGF transcriptional control Sp1 activates, whereas Sp3 inhibits, CTGF promoter activity. Previous studies have shown Sp3 down-regulates collagen gene expression in eukaryotic cells, presumably by competition with endogenously expressed Sp1 (Ghayor et al., 2001; Hagen et al., 1994). Thus the results of these studies are in keeping with observations made within this chapter.

Using EMSA I have shown the 3' putative Sp-binding site forms a DNA protein complex (Figure 5.3). A CTGF promoter reporter containing a mutation in the 3' putative Sp-binding site was insensitive to transactivation by transiently transfected Sp1. However, both the wild type CTGF promoter and one containing a mutation in the 5' Sp-binding site were transactivated (Figure 5.6), confirming Sp1 transactivation of the CTGF promoter is mediated via the 3' Sp-binding site. Consistent with the role of Sp1 in controlling basal transcriptional expression, activity of the CTGF promoter/reporter construct with a 3' Sp-binding site mutation was reduced (Ghayor et al., 2001; Willmann and Trueb, 1994; Kingsley and Winoto, 1992). These data suggest

that Sp1 is important in the basal regulation of CTGF within normal fibroblasts, acting via a functional Sp-binding site 3' to the TATA box.

To assess the contribution of Sp-binding in the elevated level of CTGF expression in SDF, these cells were transfected with either the full-length wild-type CTGF promoter/reporter construct, or with an otherwise identical construct with a mutation in its functional 3' Sp-binding site (Figure 5.6). Mutation of the 3'Sp-binding site resulted in a 50% decrease of basal CTGF promoter activity in SDF. Similarly, incubation of cells with the Sp-specific inhibitor, mithramycin (Ihn and Tamaki, 2000; Blume et al., 1991), reduced elevated CTGF promoter activity (Figure 5.7) and protein expression (Figure 5.8). This is consistent with CTGF regulation being, primarily, transcriptionally controlled (Grotendorst et al., 1996). Collectively, the results support the hypothesis that the constitutive over-expression of CTGF in SDF is Sp-dependent.

Several studies have shown Sp1 and Sp3 to have antagonistic effects (Birnbaum et al., 1995; Ghayor et al., 2001). Indeed, the co-expression studies presented in this chapter found Sp3 to inhibit Sp1 transactivation of the CTGF promoter (Figure 5.5). One study suggests that competition for Sp-binding sites by Sp3 results in the repressive effects exhibited by this Sp1-like family member (Hagen et al., 1994). No difference, as determined by Western blot in the ratio of Sp1 and Sp3 expression, was observed in SSc and control cell lines (Figure 5.9)

To assess whether the elevated levels of CTGF observed in SDF was a consequence of an elevated level of Sp1 binding, the total level of cellular Sp binding to a Sp-binding element was evaluated by EMSA. Nuclear proteins from SDF exhibited elevated Sp-binding to consensus oligonucleotides compared to healthy controls (Figure 5.10A). Elevated Sp1 DNA binding potential was determined by enzyme-linked immunosorbent assay (Figure 5.9). According to either assay, subjects with SSc possessed significantly elevated levels of Sp-binding, especially Sp1 binding, relative to healthy subjects (Figure 5.9). In addition to Sp1 and Sp3 other Sp1-like/KLF family members effectively bind to a consensus Sp-binding site (Kaczynski et al., 2003; Kingsley and Winoto, 1992). It is possible that the elevated Sp-binding activity in SSc nuclear extracts could be caused by other members of the Kruppel-like Sp1 family, and that these members show elevated expression in lesional diffuse SDF, relative to normal fibroblasts. Although Sp1 activity is elevated in SSc nuclear extracts, as measured by EMSA, the

precise identity of the proteins binding to the CTGF promoter may include other members of this extensive family of transcription factors. Confirmation of the composition of the Sp-DNA complex by supershift or transfection of siRNA against Sp1 would clarify specifically if Sp1 was primarily responsible for the elevated levels of CTGF observed in SDF.

Functional Sp1 binding motifs have been found in the minimal promoters of several collagen genes (Willmann and Trueb, 1994; Nehls et al., 1991; Tamaki et al., 1995; Rossouw et al., 1987). For example, mithramycin inhibits collagen $\alpha 1$ type I protein expression in normal fibroblasts, and Sp1 has been shown to activate expression of this gene's promoter (Greenwel et al., 1997). Furthermore, analysis of the collagen $\alpha 2$ type I promoter has shown that TGF- β activates its expression through a complex process involving SMAD3, -4 and Sp1 (Zhang et al., 2000). Interestingly, in these studies, we found mithramycin inhibited TGF- β induction of CTGF expression in normal fibroblasts (Figure 5.7). Previous reports have shown Sp1 to interact with Smads to facilitate Smad/DNA interactions with several different promoters, including COL1A2 (Docagne et al., 2004; Greenwel et al., 1997; Kumar and Butler, 1998; Zhang et al., 2000). As discussed in Chapter 4, differences in reports relating to the activity of Smads may result from examination of different disease stages. Studies in the late 1990's identified a region in the COL1A1 promoter containing Sp-binding sites that were required for the elevated expression in scleroderma (Hitraya et al., 1998).

Sp/Sp1 has been shown to directly contribute to the expression of a wide variety of matrix genes (Verrecchia et al., 2001b). However the studies presented in this chapter have sought to address the role of Sp/Sp1 in the regulation of fibrogenic genes, in a fibrotic setting. Sp1 or other family members may contribute directly to the elevated level of pro-fibrotic gene expression in fibrosis. The studies presented within this chapter suggest the mechanism of constitutive CTGF over-expression in SDF is a result of elevated promoter activity through the Sp-binding site. In addition, SDF display elevated functional Sp1/DNA binding activity. Sp1 generally acts with other transcription factors to potentiate transcription (Zhang et al., 2000; Chamboredon and Castellazzi, 2005; Czuwara-Ladykowska et al., 2001b; Sanchez-Elsner et al., 2004) so it is likely that Sp1 acts with these factors to activate transcription of genes, such as CTGF, expressed in SDF. CTGF has been reported to be an effective marker of fibrosis (Sato et al., 2000), and recent reports suggest elevated expression of collagen in SDF

may also be dependent on Sp-binding (Ihn and Tamaki, 2000). Sp1 is involved in the general basal regulation of genes, as well as facilitating the interaction of transcription factors, such as Smads, in forming transcription factor complexes to promoters. Thus the 'fibrogenic' phenotype of the SDF in established areas of fibrosis, may well result from dysregulation of basal Sp1, or from the recruitment of other factors by activated Sp1 binding. The mechanisms by which Sp1 becomes activated will be addressed in the next chapter.

5.6 Summary

The studies presented within this chapter have investigated the regulatory elements within a region of the proximal promoter sufficient to confer the high levels of CTGF promoter activity observed in SDF. Analysis of this promoter region has identified a functional Sp-binding site 3' of the TATA box. Inhibition of Sp-DNA binding or deletion of this functional site reduces excessive CTGF promoter activity in SDF. Consistent with this Sp1 induces CTGF promoter activity via this Sp-binding site, whereas Sp3 inhibits Sp1 effects in normal fibroblasts. Although expression levels of Sp1 and Sp3 appears unaltered in SDF, SDF nuclear extracts exhibit elevated Sp-binding activity to consensus DNA binding sequences and increased levels of Sp1-DNA binding activity.

Chapter 6:

ERK mediates CTGF over-expression in SSc fibroblasts via Sp-binding

6.1 Introduction

Sp1 is the founding member of the Sp1-like/KLF family of transcription factors. The studies presented in this chapter will explore the mechanism underlying the enhanced Sp1-DNA binding activity in SDF, and the relevance to over-expression of CTGF in these cells. In Chapter 5 I demonstrated that, although Sp3 inhibited the ability of Sp1 to activate CTGF, the total protein levels of both were not significantly different in SSc, suggesting that differences in the activation state of Sp1 may be due to regulation of protein/DNA interactions. Transcriptional activity of Sp1 is predominantly regulated through post translational modifications such as phosphorylation.

6.1.1 Post-translational modifications of Sp1

Post-translational modification of Sp1 alters the binding affinity for DNA. Sp1 is post-transcriptionally modified via glycosylation (Jackson and Tjian, 1988), acetylation (Ryu et al., 2003) and phosphorylation (Borellini et al., 1990; Jackson et al., 1990). Glycosylation of multiple potential *O*-glycosylation sites by N-acetylglucosamine residues appears to protect Sp1 from proteasome degradation (Han and Kudlow, 1997; Jackson and Tjian, 1988). The precise role of acetylation in governing Sp1 function remains unclear, being cell type specific and has positive and negative effects on Sp1 activities (Hilton et al., 2005; Gan et al., 2005). Phosphorylation of Sp1 was first reported in 1990 (Jackson et al., 1990) and has subsequently led to multiple Sp1 phosphorylation sites being identified (Midgley and Khachigian, 2004; Bonello and Khachigian, 2004; Chun et al., 1998; Armstrong et al., 1997). Known sites of glycosylation and phosphorylation that have been identified in Sp1 are shown in Figure 6.1.

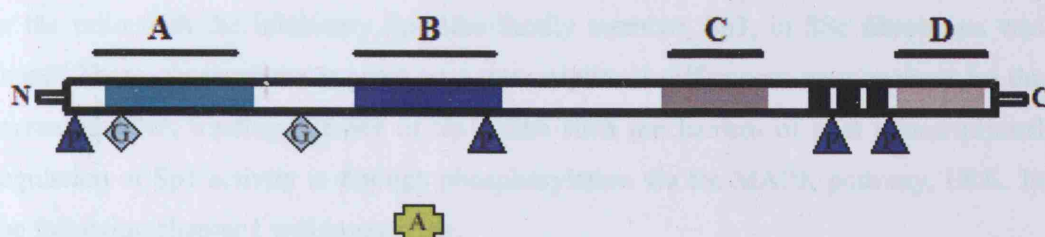


Figure 6.1

Regions of post-transcriptional modification of Sp1.

Phosphorylation (P) and glycosylation (G) sites are as indicated. Acetylation (A) sites are not known. The black bars marked A, B, C and D denote Sp1 sub-domains.

6.1.2 Sp1 phosphorylation

Phosphorylation is a widely occurring rapid mechanism of regulating the function of proteins (Hayashida et al., 2003; Hocevar et al., 1999; Bai et al., 2004; Ihn et al., 2001a; Mori et al., 2004). Phosphorylation of Sp1 occurs due to a variety of circumstances and stimuli including environmental changes, such as viral infection (Chun et al., 1998), in the presence of growth factors such as FGF-2 and TGF- β (Bonello and Khachigian, 2004; Greenwel et al., 1997) and during cell division (Black et al., 1999). Multiple kinases have been shown to phosphorylate Sp1, including cyclin-dependent kinase 2 involved in the cell cycle (Haidweger et al., 2001), growth-related kinase (Black et al., 1999) and the growth factor induced MAPK, ERK (Bonello and Khachigian, 2004; Merchant et al., 1999). In turn, de-phosphorylation by phosphatases controls the balance of action of these kinases and thus maintains the Sp1 phosphorylation *status quo* in the cell. For example, okadaic acid promotes Sp1 phosphorylation by inhibiting the action of protein phosphatase 1 (Wang et al., 1999). Thus, the steady state level of Sp1 phosphorylation is likely to be a dynamic balance between the activities of kinases and phosphatases. The cumulative effect of the broad ranges of kinases and phosphatases that act on Sp1 allows for a wide range of signal pathways to transmit pathophysiological signals to target genes via changes in the phosphorylation of Sp1 and its ability to interact with Sp-DNA binding sites (Armstrong et al., 1997; Chun et al., 1998; Greenwel et al., 1997; Midgley and Khachigian, 2004; Wang et al., 1999).

6.2 Aims

The mechanism by which Sp1 is regulated is perhaps the best understood of all the Sp1-like/KLF family. In the previous chapter, SSc fibroblasts were found to possess increased Sp1/DNA binding activity. However, no difference in the total levels of Sp1 or the ratio with the inhibitory Sp1-like family member, Sp3, in SSc fibroblasts was found. These observations suggest post-transcriptional differences may account for the increased DNA binding activity of Sp1. One such mechanism of post transcriptional regulation of Sp1 activity is through phosphorylation via the MAPK pathway, ERK. In the following chapter I will investigate:

- 1- Expression of specific components of the MAPK pathway in SSc fibroblasts.
- 2- The effect of blockade of these pathways on Sp-binding activity and CTGF expression in SSc.

6.3 Methods and Methods

6.3.1 Cell culture

Control and SDF cell lines, previously characterised as expressing high levels of CTGF (Chapter 3) were used in the course of the studies presented within this chapter. All primary dermal fibroblast cell lines, HFF, and NIH/3T3, were maintained in FGM media as described in Section 2.2. For experiments involving serum stimulation, cells were maintained in FGM media supplemented with 0.2% FCS for 48 h prior to serum stimulation in FGM media supplemented with 15% FCS.

6.3.2 Protein Kinase inhibitors

The following inhibitors were used in this study: U0126 (Promega, UK) a MEK inhibitor which is non-competitive with respect to MEK substrates, ATP and ERK1/2 (Favata et al., 1998). SP600125 (Calbiochem) is soluble in DMSO and is a selective, reversible inhibitor of c-Jun N-terminal kinase (JNK) ($IC_{50} = 40$ nM for JNK-1 and JNK-2 and 90 nM for JNK-3). The inhibition is competitive with respect to ATP, exhibiting over 300-fold greater selectivity for JNK as compared to ERK1 and p38 MAPK. SP600125 acts by inhibiting the phosphorylation of c-Jun (Bennett et al., 2001). All inhibitors were dissolved to a desired stock concentration and further dilutions were prepared in sterile DMEM unless otherwise stated and carrier alone added as appropriate. Cells were treated at previously published doses (Favata et al., 1998; Shiwen et al., 2006a).

6.4 Results

6.4.1 Dysregulated expression of MAPK in SSc.

In the previous chapters, a promoter region between -86 and +17 bp has been shown to encompass a functional Sp1 site and confer the constitutive over-expression of reporter gene when transiently introduced into SSc fibroblasts. Sp1 activity is regulated by several mechanisms, including phosphorylation by members of the MAPK pathway. Therefore to investigate this, expression of JNK, ERK1/2, p38 and CTGF were determined in total protein extracts from two control and six SDF by Western blot. In keeping with previous results, SDF constitutively expressed CTGF. No difference in total levels of JNK1/2, ERK1/2, p38 or phospho-p38 in SSc was found. In contrast, levels of phospho-JNK1 and phospho-ERK1/2 were consistently elevated in SSc dermal fibroblasts. (Figure 6.2 left panel). Further, whereas TGF- β treatment of normal fibroblasts did not affect total levels of JNK, ERK1/2 and p38, phosphorylation of all three MAPKs was elevated (Figure 6.2 right panel).

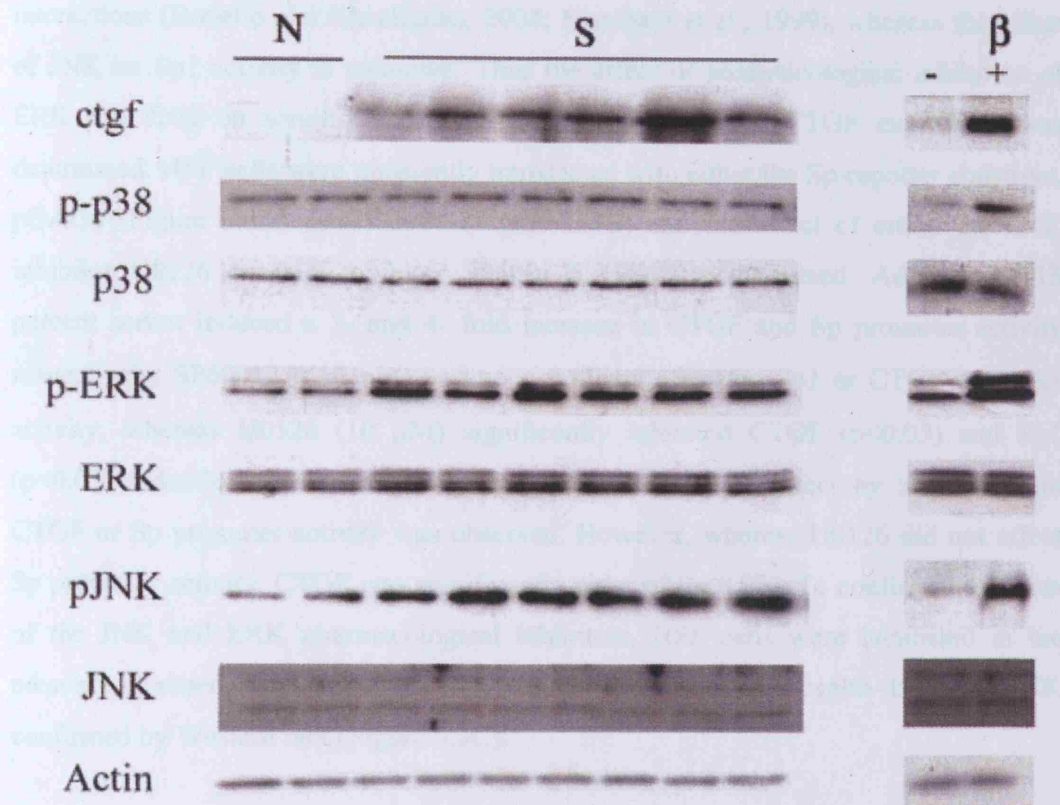


Figure 6.2

Elevated phosphorylated ERK1/2 and JNK1 in SDF.

Control and SDF were grown to confluence and serum starved for 24 h. Total and phosphorylated levels of p38, ERK1/2 and JNK1/2 and the levels of CTGF and actin (loading control) in the cell layer were determined by Western blotting (left panel). MAP kinases expression in normal fibroblasts in the presence and absence of TGF-β1 (β) (right panel).

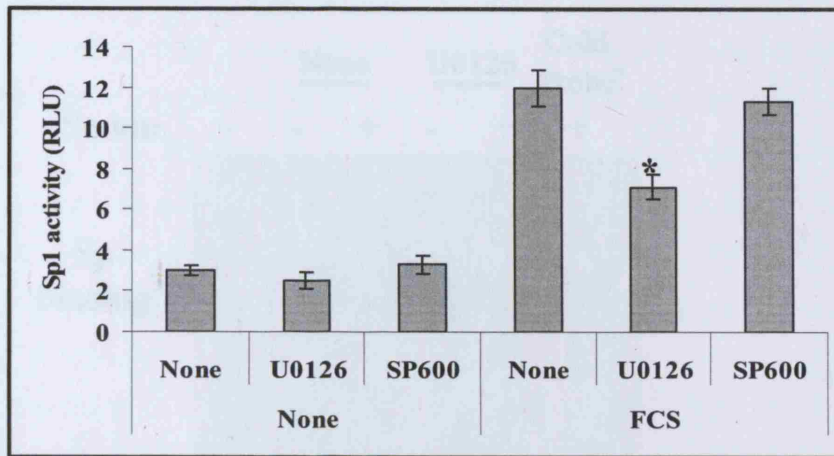
6.4.2 Serum induction of Sp and CTGF reporter gene activity is in part mediated via ERK1/2

Previous reports have shown Sp1 DNA binding to be induced by serum (Kumar and Butler, 1998). In addition, ERK is known to phosphorylate Sp1 and promote Sp1/DNA interactions (Bonello and Khachigian, 2004; Merchant et al., 1999), whereas the effect of JNK on Sp1 activity is unknown. Thus the effect of pharmacological inhibition of ERK and JNK on serum-induced Sp-binding to DNA and CTGF expression was determined. HFF cells were transiently transfected with either the Sp-reporter construct, pGAG6 (Figure 6.3A) or CTGF86 (Figure 6.3B) and the effect of either the ERK inhibitor U0126 or JNK inhibitor SP600125 (SP600) determined. Addition of 15 percent serum induced a 3- and 4- fold increase in CTGF and Sp promoter activity respectively. SP600125 (10 μ M) had no significant effect on Sp1 or CTGF promoter activity, whereas U0126 (10 μ M) significantly inhibited CTGF ($p<0.05$) and Sp1 ($p<0.05$) induction by serum. In serum-free conditions, no effect by SP600125 on CTGF or Sp promoter activity was observed. However, whereas U0126 did not affect Sp promoter activity, CTGF was significantly reduced ($p<0.05$). To confirm the effects of the JNK and ERK pharmacological inhibitors, HFF cells were incubated in the presence or absence of SP600125 or U0126 and expression of phospho- ERK and JNK confirmed by Western blot (Figure 6.3C).

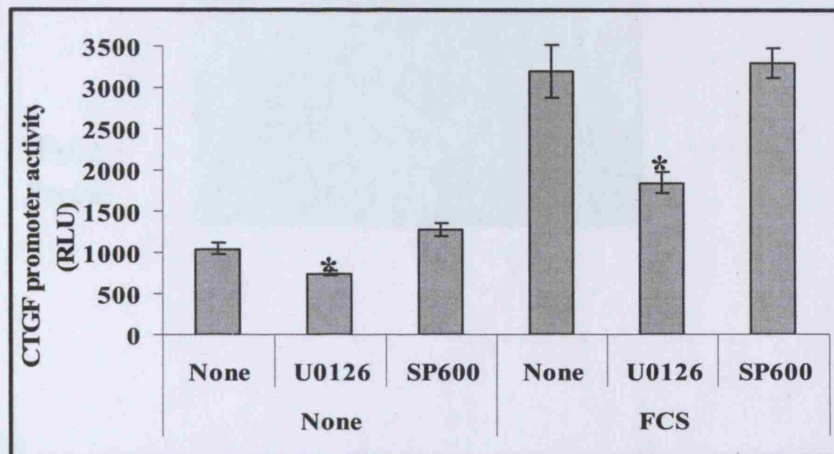
6.4.3 U0126 inhibits serum induced Sp-binding to the CTGF promoter

Serum-starved HFF cells were stimulated in the presence or absence of 15% serum and nuclear extracts isolated as previously described (Section 2.5.1). The pharmacological inhibitor of ERK, U0126 (10 μ M), was added to medium 2 hours prior to serum stimulation. To assess the effect on Sp-binding to the CTGF promoter, nuclear extracts were isolated and EMSA performed. Serum induced an increased binding to the CTGF promoter (Figure 6.4 lane 3 vs lane 2). Inhibition of ERK activation by U0126 did not affect un-stimulated Sp-binding to the CTGF promoter (Figure 6.4 lane 4), whereas serum induced binding was reduced by U0126 (Figure 6.4 lane 5 vs 3). Competition with an equal-molar concentration of un-labelled CTGF probe reduced Sp-binding (Figure 6.4 lane 6).

A



B



C

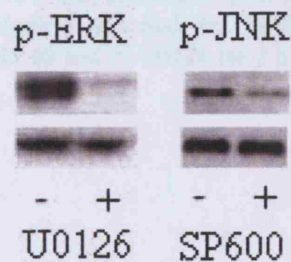


Figure 6.3
Inhibition of ERK but not JNK, represses serum-induced Sp1 activity and CTGF expression in normal fibroblasts.

HFF fibroblasts were grown to confluence and transiently transfected with either the Sp1 reporter construct pGAG6 (A) or the CTGF promoter/reporter construct CTGF86 (B) in the presence or absence of U0126 (10 μ M) or SP600125 (10 μ M) in serum-free conditions. Media was replaced with DMEM containing either 0.2% (None) or 15% foetal calf serum (FCS), and reporter gene activity measured after 24h. SEAP and luciferase activity was normalized for transfection efficiency by β -galactosidase activity via co-transfection with CMV- β -gal. Data shown is representative of 2 experiments with a total of n=6 wells; mean \pm SEM, *p<0.05, students unpaired t-test. Inhibition of phospho-ERK or JNK by U0126 or SP600125 was confirmed by Western blot (C).

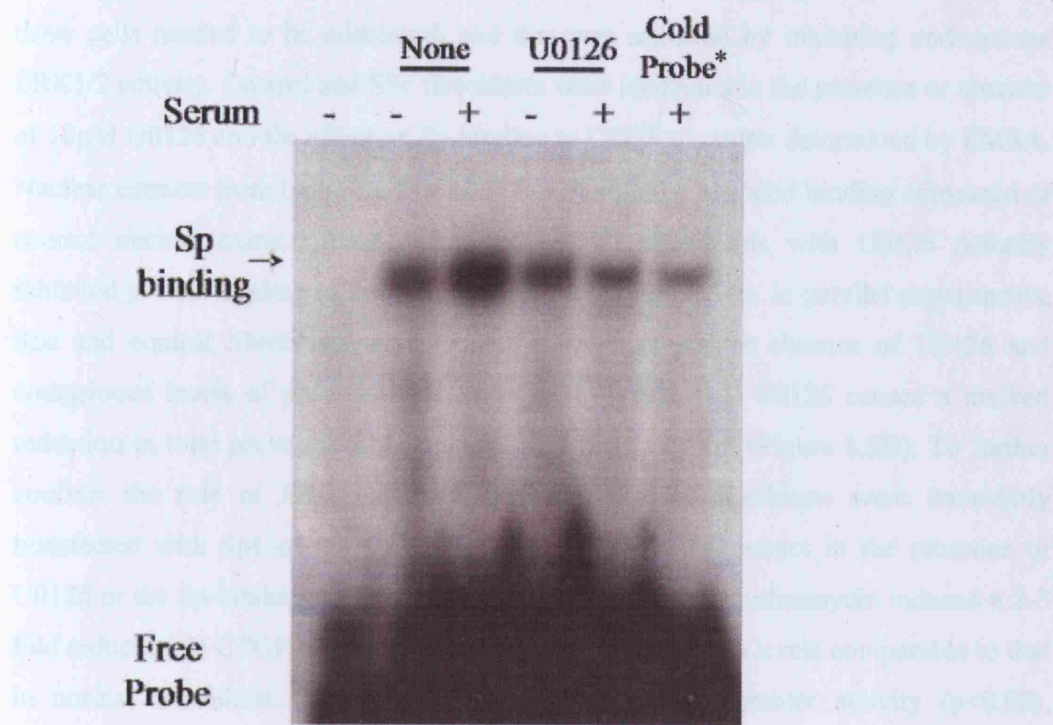
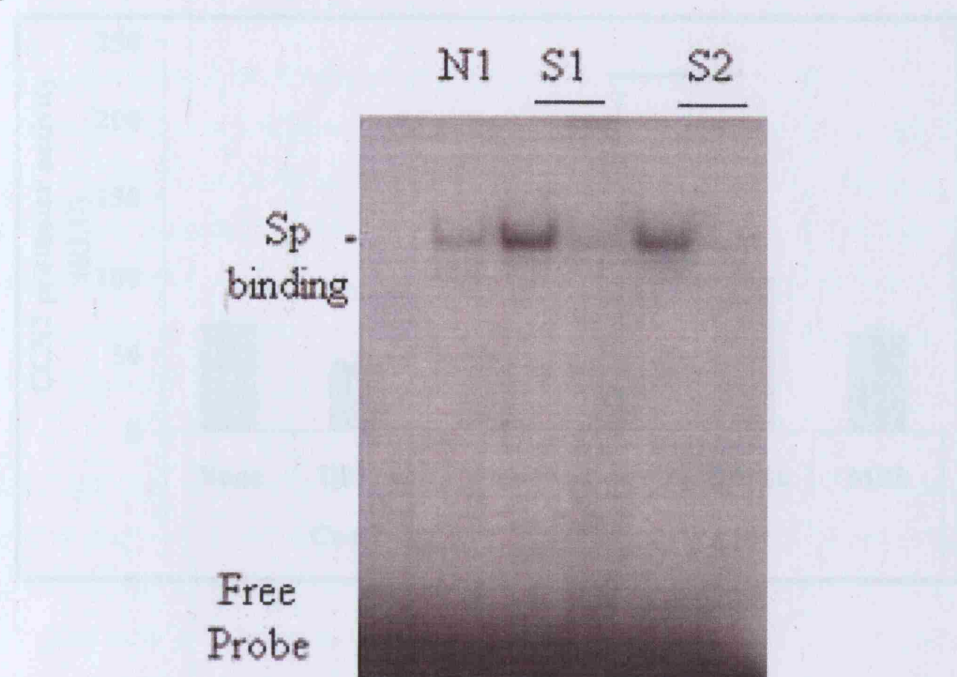


Figure 6.4
Effect of ERK1/2 repression on serum induced Sp-binding to the CTGF promoter.
 HFF cells were serum starved for 48 h and stimulated with 15% serum (FCS) for 4 h. Nuclear extracts were isolated and Sp-binding assessed by EMSA. For studies involving ERK repression, cells were incubated with 10 μ M of U0126 for 2 h prior to serum stimulation.

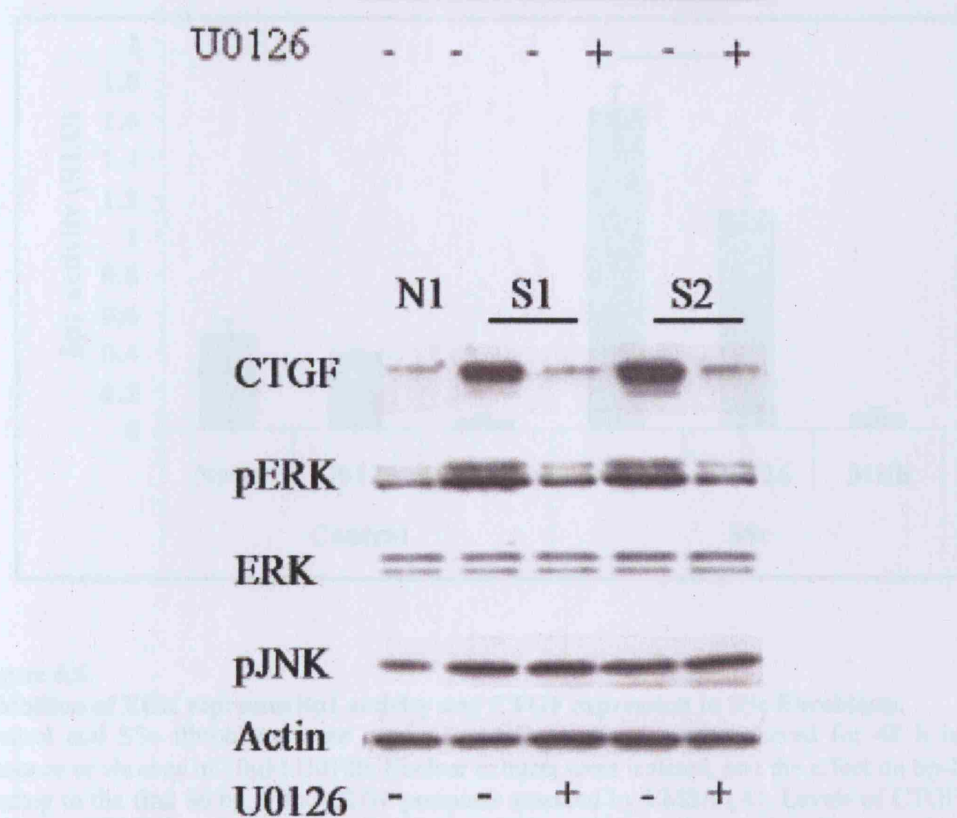
6.4.4 Role of ERK in constitutive CTGF expression and Sp-binding in SDF

The requirement of ERK in SSc fibroblasts for constitutive expression of CTGF within these cells needed to be addressed, and this was achieved by inhibiting endogenous ERK1/2 activity. Control and SSc fibroblasts were incubated in the presence or absence of 10 μ M U0126 and the effect on Sp-binding to CTGF promoter determined by EMSA. Nuclear extracts from both SSc fibroblast lines exhibited elevated binding compared to control nuclear extract. Prior incubation of SSc fibroblasts with U0126 potently inhibited protein binding to the CTGF promoter (Figure 6.5A). In parallel experiments, SSc and control fibroblasts were treated in the presence or absence of U0126 and endogenous levels of phospho-ERK and CTGF determined. U0126 caused a marked reduction in total protein levels of phospho-ERK and CTGF (Figure 6.5B). To further confirm the role of ERK and Sp1, SSc and control fibroblasts were transiently transfected with Sp1 or the CTGF86 promoter/reporter construct in the presence of U0126 or the Sp-binding inhibitor mithramycin. U0126 and mithramycin induced a 2-3 fold reduction in CTGF promoter activity in SSc fibroblasts to levels comparable to that in normal fibroblasts. U0126 significantly reduced Sp-promoter activity ($p<0.05$), whereas mithramycin completely inhibited expression (Figure 6.5C).

A



B



Protein	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6
pJNK	+	+	+	++	+	+
Actin	+	+	+	+	+	+
U0126	-	-	-	-	+	+

C

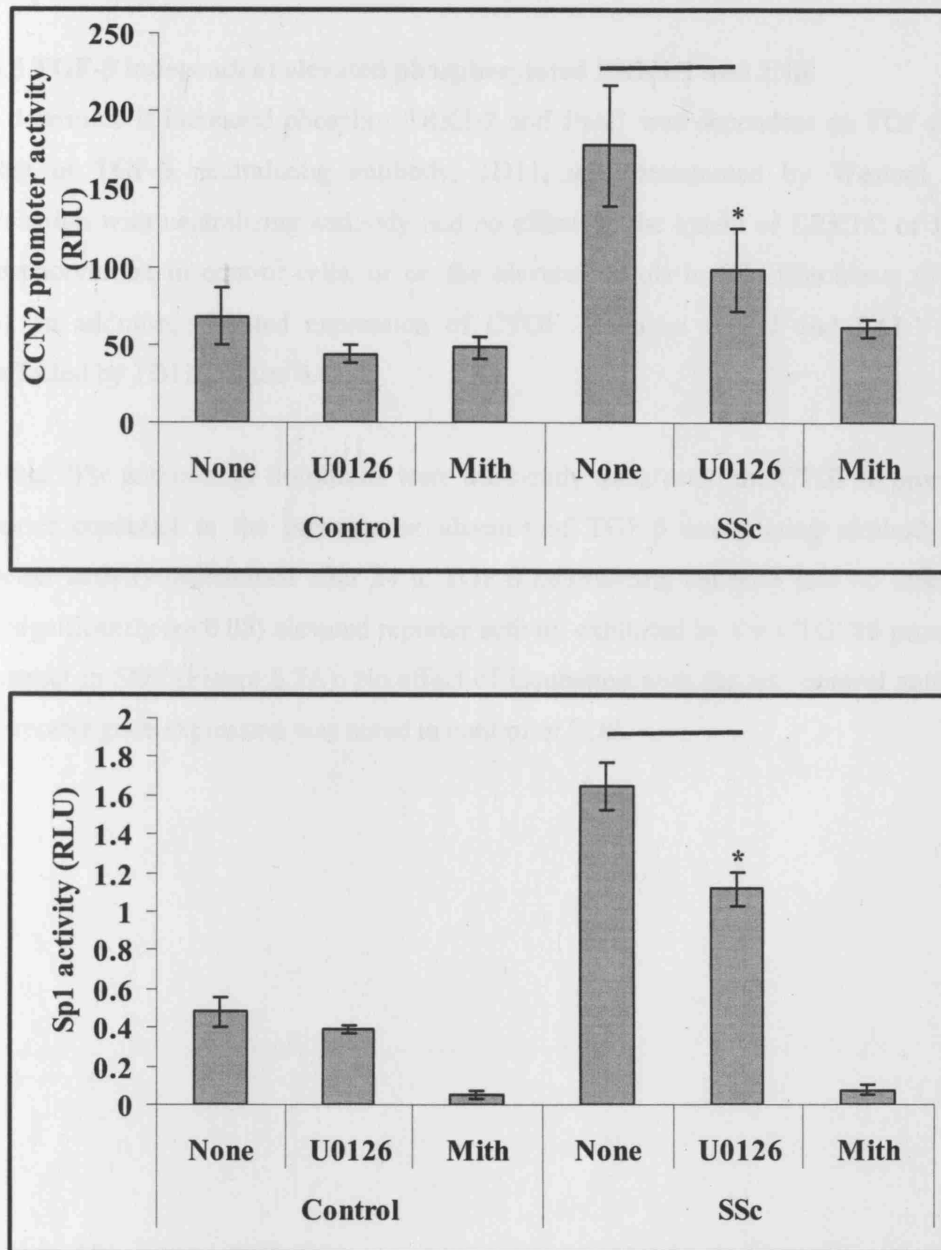


Figure 6.5

Inhibition of ERK represses Sp1 activity and CTGF expression in SSc fibroblasts.

Control and SSc fibroblasts were grown to confluence and serum starved for 48 h in the presence or absence of 10 μ M U0126. Nuclear extracts were isolated, and the effect on Sp-DNA binding to the first 86 bp of the CTGF promoter assessed by EMSA (A). Levels of CTGF and phospho- ERK1/2 and JNK were determined in parallel experiments by Western blot (B). Normal and SSc fibroblast cell lines were transiently transfected with the Sp reporter construct, pGAG6, or the CTGF promoter/reporter construct, CTGF86, in the presence or absence of 10 μ M U0126 or 150 nM Mithramycin (Mith). After 24 h incubation in serum-free media, SEAP and luciferase activity was measured and normalized for transfection efficiency (C). Data shown is representative of 3 cell lines n=3 wells; mean \pm SEM, *p<0.05, Students unpaired t-test.

6.4.5 TGF- β independent elevated phosphorylated ERK1/2 and JNK

To determine if increased phospho- ERK1/2 and JNK1 was dependent on TGF- β , the effect of TGF- β neutralizing antibody, 1D11, was determined by Western blot. Incubation with neutralizing antibody had no effect on the extent of ERK1/2 or JNK1 phosphorylation in control cells, or on the elevated levels in SSc fibroblasts (Figure 6.6). In addition, elevated expression of CTGF, Collagen type I and PAI-1 were unaffected by 1D11 (Figure 6.6).

Further, SSc and control fibroblasts were transiently transfected the CTGF86 promoter reporter construct in the presence or absence of TGF- β neutralizing antibody and reporter activity determined after 24 h. TGF- β neutralizing antibody had no effect on the significantly ($p < 0.05$) elevated reporter activity exhibited by the CTGF86 promoter construct in SDF (Figure 6.7A). No effect of incubation with the IgG control antibody on reporter gene expression was noted in control or SDF.

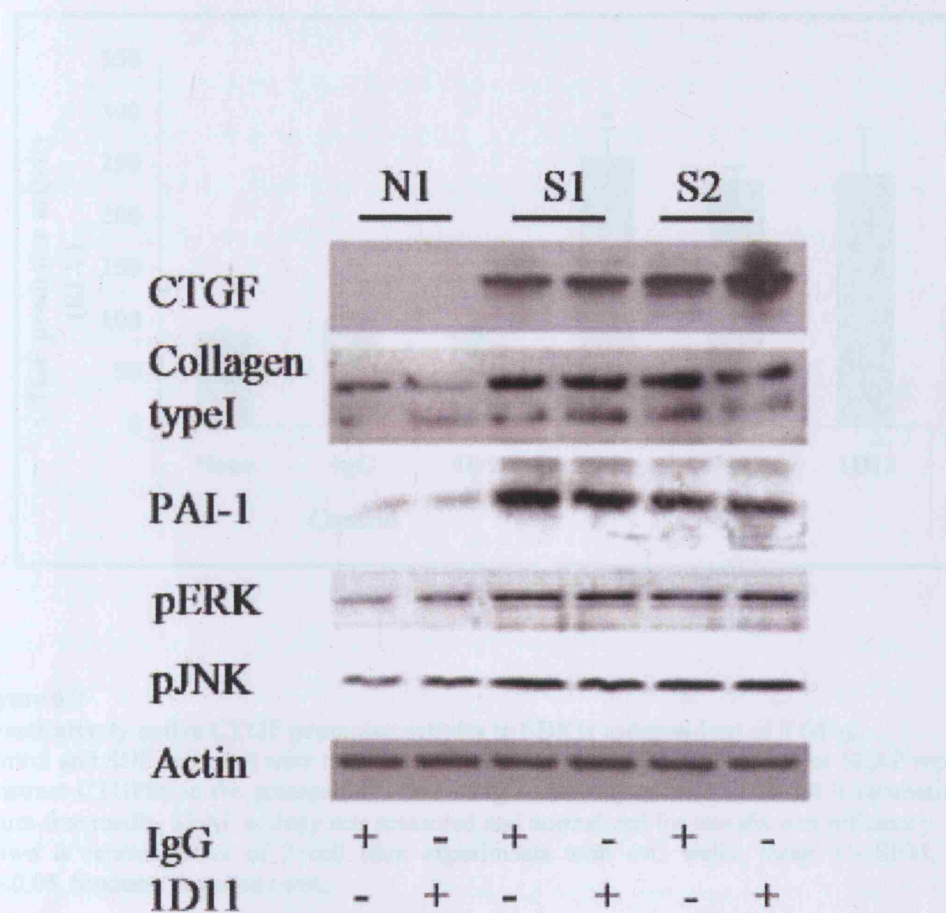


Figure 6.6
Constitutively active ERK and JNK are independent of TGF- β in SSc dermal fibroblasts.
 Control and SSc fibroblasts were grown to confluence and serum starved for 24 h in medium containing (+) or lacking (-) the TGF- β neutralizing anti-body 1D11. Total levels of CTGF, collagen type I, PAI-1 and phosphorylated levels of ERK1/2 and JNK1 in the cell layer were determined by Western blotting.

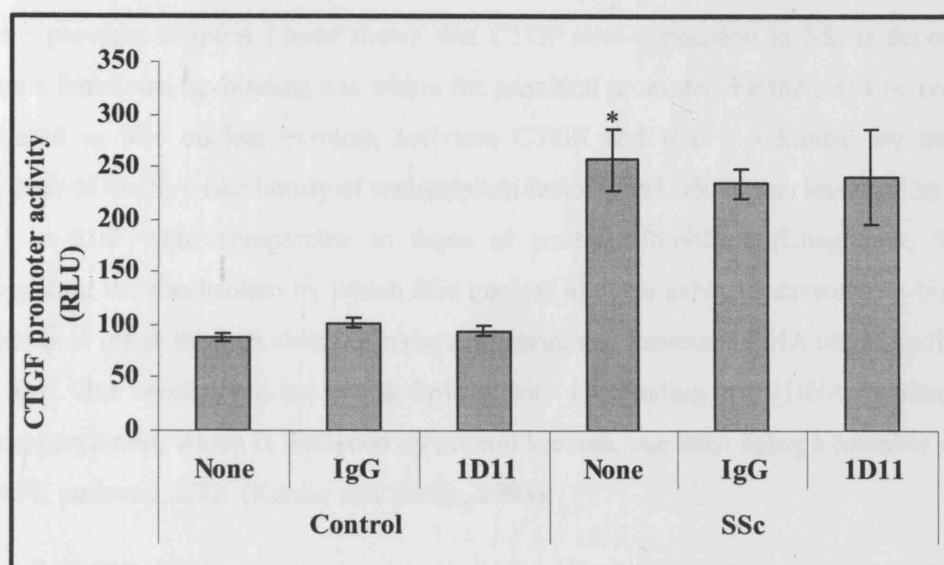


Figure 6.7

Constitutively active CTGF promoter activity in SDF is independent of TGF- β .

Control and SDF cell lines were transiently transfected with the CTGF promoter SEAP reporter construct CTGF86 in the presence of 1D11 or IgG antibody control. After 24 h incubation in serum-free media, SEAP activity was measured and normalized for transfection efficiency. Data shown is representative of 2 cell lines experiments with n=3 wells; mean \pm SEM, n=6.

*p<0.05, Students unpaired t-test.

6.5 Discussion

In the previous chapters I have shown that CTGF over-expression in SSc is dependent upon a functional Sp-binding site within the proximal promoter. Further, Sp1 activation, elevated in SSc nuclear extracts, activates CTGF and this is inhibited by another member of the Sp1-like family of transcription factors, Sp3. However, levels of Sp1 and Sp3 in SDF were comparable to those of control fibroblasts (Chapter 5; 5.4.7), suggesting the mechanism by which SSc nuclear extracts exhibit increased Sp-binding is likely to result through changes in the activation, and increased DNA binding affinity, of Sp1. One mechanism by which Sp1 affinity for binding with DNA is altered is phosphorylation, which is mediated by several kinases, one such being a member of the MAPK pathway, ERK (Kumar and Butler, 1998).

To address the role of the MAPK pathway in constitutive expression of CTGF, the expression of three members of the family JNK, ERK and p38 were investigated. Protein levels of both phosphorylated JNK1 and ERK1/2 were significantly elevated in SSc dermal fibroblasts (Figure 6.3). During the course of these studies Trojanowska's group, and others, have reported elevated phospho-ERK in pulmonary lung and dermal fibroblasts, from SSc patients (Svegliati et al., 2005; Tourkina et al., 2005). Interestingly, the Trojanowska group have previously reported no difference in p38 levels in SDF (Sato et al., 2002), consistent with the finding presented here (Figure 6.3). In contrast, another report has found constitutive phosphorylation and activation of p38 MAPK in SDF (Ihn et al., 2005). Intriguingly both groups investigated the effects of pharmacological inhibition of p38, reporting opposing effects on constitutive expression of collagen by SSc fibroblasts. Indeed, a recent study found elevated ERK activation in SDF from involved fibrotic regions but no increased phospho-JNK, in contrast to the results presented within this chapter (Svegliati et al., 2005). The data presented within this chapter is the first to assess the relative activation status of all three MAPK pathways simultaneously, and to relate this to the expression of a functionally relevant gene. The apparent differences in activation status of the MAPK pathways may represent stage specific differences in the progression of this heterogeneous disease.

To determine the functional relevance of constitutive phosphorylation and activation of the JNK and ERK pathways on CTGF expression, the effect of pharmacological inhibitors on serum-induced CTGF was determined. In Chapter 5 the Sp-binding site in the CTGF promoter was found to play a critical role in CTGF over-expression in SSc

fibroblasts, and other studies have shown Sp1 activity to be up-regulated in serum-stimulated fibroblasts (Kumar and Butler, 1998). In addition, the mouse homologue of CTGF, fisp-12, was originally identified as a serum-induced gene in NIH/3T3 cells (Ryseck et al., 1991). Thus, using the minimal promoter region that confers the elevated reporter activity of CTGF in SSc fibroblasts, the effect of serum was investigated. CTGF promoter activity was upregulated in response to serum (Figure 6.5B), consistent with the mapping of a functional Sp-binding site within this region of the promoter. In addition the reporter activity of the Sp-reporter construct, pGAG6, was elevated (Figure 6.5A). This confirms that both Sp1 and CTGF promoter activity was elevated in response to serum. Pharmacological inhibitors of ERK, but not JNK, significantly inhibited this effect (Figure 6.5A/B) in keeping with previous observations (Bonello and Khachigian, 2004; Merchant et al., 1999). Thus serum-induced CTGF expression is dependent on ERK activation of Sp-binding.

TGF- β activates other distinct signalling pathways in addition to the specific Smads, including the MAPK pathways JNK, ERK and p38 (Holmes et al., 2001; Leask et al., 2003; Chen et al., 2000; Sato et al., 2002) (Figure 6.3 right panel). In this chapter it was determined that activation of the CTGF promoter, between -86 and +17 bp, which contains a functional Sp1 site, is dependent on ERK activation. Blockade of TGF- β using neutralizing antibodies did not affect the constitutively activated ERK or JNK observed in SSc fibroblasts, or the elevated Sp1 or CTGF reporter activity exhibited by SSc fibroblasts (Figure 6.5). This supports the notion that activation of the MAPK pathways, JNK and ERK, and Sp1, in SSc fibroblasts is independent of TGF- β ligand. In addition, over-expression of CTGF, and two further proteins previously reported as elevated in SSc fibroblasts and inducible by TGF- β in normal cells, were un-affected by neutralizing TGF- β antibody 1D11. Thus the precise role of TGF- β , at least in maintaining the *in vitro* fibrotic phenotype of SSc fibroblasts, as determined by the expression of collagen type I, PAI-1 and CTGF, appears to be less than clear. The MAPK pathway, in addition to TGF- β , is activated by environmental changes, such as mechanical stretch, and numerous factors implicated in scleroderma including ET-1 and PDGF (Gao et al., 2005; Xu et al., 2004). Asano *et al.* recently demonstrated increased expression of integrins $\alpha_v\beta_3$ in scleroderma fibroblasts (Asano et al., 2005). Intriguingly, CTGF binds these same integrins (Gao and Brigstock, 2004), and facilitates the activation of ERK (Chen et al., 2004). Thus constitutive expression of CTGF in SSc fibroblasts may be a result of over-expression of the $\alpha_v\beta_3$ integrins and

autocrine induction of itself. Recent studies demonstrated that conditioned media from SSc fibroblasts were able to promote over-expression of collagen type I and CTGF, in normal fibroblasts (Zhou et al., 2005b). Taken together, these observations support the hypothesis that secretable factor(s) play a key role in maintaining the fibrotic phenotype. Determining the factor(s) that confer this affect remains to be achieved.

Although the studies presented in this chapter demonstrate CTGF over-expression is independent of JNK, the physiological effect of constitutive activation of JNK on SSc fibroblast function remains unclear. For example, in normal cells JNK has been shown to be critical for *in vitro* models of wound closure (Javelaud et al., 2003) and in the induction of matrix genes including fibronectin (Hocevar et al., 1999), which is elevated in the dermis of SSc patients (Rajkumar et al., 2005). Collectively, these studies show activation of the MAPK pathways is independent of TGF- β , and that other factors including CTGF itself, may be responsible for the activation of either JNK ERK, or both, within the disease context. Clearly, further investigations are needed to identify the mechanisms by which these pathways are activated.

6.6 Summary

In summary, the studies presented within this chapter have shown SDF exhibit constitutive activation of the JNK1, ERK1/2 but not p38 signalling pathways. The constitutive activation of these members of the MAPK pathway are independent of TGF- β ligand. Furthermore, inhibition of ERK1/2 suppresses over-expression of CTGF by SDF via repression of Sp1-like DNA binding to the CTGF promoter, supporting the notion that dysregulation in the MAPK pathways is essential for the over-expression of CTGF in SDF.

Chapter 7:

General discussion and future studies

Over-expression of CTGF is a cardinal feature both of animal models of fibrosis and human fibrotic pathologies such as Systemic Sclerosis (SSc). The development of therapeutics for fibrotic diseases is likely to depend on a better understanding of the underlying molecular mechanisms controlling this process. CTGF represents both a marker and putative mediator of fibrosis. The general aim of the studies presented here was to investigate the molecular mechanisms underlying the over-expression of CTGF in SSc dermal fibroblasts (SDF). In this chapter, I will summarise and discuss the results presented in Chapters 3, 4, 5 and 6. In addition I will discuss the putative functional role of CTGF in SSc.

7.1 Regulation of CTGF by TGF- β

TGF- β is a potent transcriptional regulator of a wide variety of genes, including those encoding fibrogenic proteins (Verrecchia et al., 2001a). Acting via specific cellular receptors, TGF- β activates both Smad and non-Smad signalling cascades to elicit downstream actions. Although the identification of the generic, non-Smad signalling pathways preceded the discovery of Smads, subsequent studies have found the majority of TGF- β transcriptional effects on genes to be primarily regulated by Smads (Yang et al., 2003). Concurrent with the identification of Smads, and their role in the regulation of TGF- β responsive genes, Grotendorst *et al.* identified a novel TGF- β responsive sequence (T β RE) within the proximal promoter region of CTGF. Deletion of this region rendered a CTGF promoter reporter construct insensitive to TGF- β induction in NIH/3T3 cells, human dermal fibroblasts and aortic smooth muscle cells (Grotendorst et al., 1996).

The primary aim of Chapter 3 was to determine if the Smad signalling pathway was involved in the induction of CTGF by TGF- β , in normal fibroblasts. An initial *in silico* analysis of the CTGF promoter revealed that the T β RE sequence (GTGTCAAGGGGTC) shared little sequence homologue to known Smad binding sites; rather several regions within a 4.5Kb region of the CTGF promoter had a high degree of homology with a Smad3 consensus DNA-binding sequence, including a sequence 13 bp 5' to the previously reported TGF- β response element. To determine the biological role of Smads in TGF- β induction of CTGF, a promoter reporter based approach was taken.

A series of promoter deletion and point mutation constructs identified a region within the CTGF promoter, distinct from the previously characterised TGF- β response element, which was critical for TGF- β induction of CTGF. This region contained a sequence which shared significant homology to a SMAD3 consensus binding site. Over-expression of Smad3, but not Smad2, transactivated the CTGF promoter reporter construct, suggestive of TGF- β induction of CTGF being mediated via Smad3. EMSA confirmed a complex, comprised of Smad3 and Smad4, formed on this Smad DNA-binding site (Holmes et al., 2001). Subsequent studies by several groups have demonstrated the requirement of Smads in activation of CTGF by TGF- β in a variety of cell types, including smooth muscle cells and mesangial cells (Fu et al., 2001; Chen et al., 2002; Leivonen et al., 2005; Phanish et al., 2005).

CTGF is rapidly transcriptionally induced by TGF- β , and consistent with this being regulated through Smad3/Smad4, microarray studies by Yang *et al.* report that Smad3 DNA-binding site motifs are a characteristic of immediate early genes. Furthermore, this study suggests that Smad2 and ERK1/2 predominantly act to modulate the actions of TGF- β /Smad3 on immediate early and intermediate genes (Yang et al., 2003). Studies within this laboratory, in addition to those of other groups, have implicated members of the MAPK pathway in the regulation of CTGF. In the majority of cell types tested thus far, ERK has been found to be critical for the induction of CTGF by TGF- β s (Chen et al., 2002; Leivonen et al., 2005; Xie et al., 2005; Phanish et al., 2005; Stratton et al., 2002), whereas over-expression of JNK1 represses the induction of CTGF by TGF- β in NIH/3T3 (Leask et al., 2003), and human dermal fibroblasts (Shi-wen *et al.* personal communication). By contrast Xie has reported the induction of CTGF by TGF- β in airway smooth muscle cells is dependent on both JNK and ERK (Xie et al., 2005), whereas in human lung fibroblasts only JNK is required (Utsugi et al., 2003). This suggests the mechanisms by which CTGF is induced by TGF- β may be cell type specific and regulated by members of the MAPK signalling pathways.

In Chapter 3 it was further demonstrated that CTGF induction by TGF- β is significantly impaired in mouse embryonic fibroblasts lacking Smad3. The activation of genes by Smads often requires co-factors (Zhang et al., 2000; Nishihara et al., 1998). However, induction of CTGF is independent of the co-factor p300 (Holmes et al., 2001). Subsequent studies performed within this laboratory have identified additional elements and transcription factors critical for the induction of CTGF by TGF- β . Deletion of a

tandem repeat of the transcription enhancer factor (TEF) binding element, 5'-GAGGAATGG-3', renders the CTGF promoter unresponsive to activation by TGF- β (Leask et al., 2003). Recently this study has been extended and members of the Ets family of transcription factors have been shown to be important in the regulation of CTGF (Nakerakanti et al., 2006; Van Beek et al., 2006). Three ETS consensus binding sites, GGA(A/T), have been identified in the CTGF promoter (Nakerakanti et al., 2006), including two contained within the previously reported TEF elements (Leask et al., 2003). Both studies verified the opposing actions of two Ets family members in CTGF transcription; whereas Ets1 activates, Fli1 represses (Nakerakanti et al., 2006; Van Beek et al., 2006). Collectively these studies suggest that TGF- β treatment leads to a reduction in the interaction of Fli1 with the CTGF promoter (Nakerakanti et al., 2006) and Ets1 synergises with Smad3 to promote a robust induction of a CTGF promoter reporter construct by TGF- β (Van Beek et al., 2006). This latter observation is in keeping with several reports from Trojanowska's group and others demonstrating Ets1 complexes with Smad3, Sp1 and/or p300 in the induction by TGF- β of genes, such as COL1A2, (Jinnin et al., 2006; Czuwara-Ladykowska et al., 2001a). It remains to be determined if the transcription factors binding the ETS DNA binding sites interact with the functional Sp-binding site within the CTGF promoter.

Previous studies have also shown TGF- β induction of CTGF to be PKC dependent (Chen et al., 2002; Crean et al., 2004). Consistent with this Van Beek established that Ets1 activation of CTGF requires PKC (Van Beek et al., 2006), an observation supported by the requirement of PKC δ in the induction of COL1A2 by Ets1 (Jinnin et al., 2005). To date 27 human Ets have been identified, which function as both activators and repressors of gene transcription (Seth and Watson, 2005). In keeping with Ets factors binding to a common sequence, several other Ets family members also modulate CTGF promoter activity (Nakerakanti et al., 2006). Work by the Trojanowska group has demonstrated that in human fibroblasts TGF- β does not increase Ets1 protein levels or promote its phosphorylation (Czuwara-Ladykowska et al., 2002). Both studies over-expressed Ets1 transiently to induce CTGF and thus it remains unclear which Ets family members regulate CTGF expression *in vivo*. Since Ets proteins are down stream substrates of the MAP kinases (ERKs, p38 and JNK), it is plausible that the differences in the cellular requirement of different MAPK components for induction of CTGF by TGF- β may result from cell specific expression or activation of the ETS family members (Nakerakanti et al., 2006). Further, phosphorylation of the Smad3 linker

region by MAPKs leads to functional changes in Smad activity, including enhancing collagen I synthesis (Hayashida et al., 2003). Defining which Ets are involved in TGF- β induction of CTGF in skin and lung fibroblasts and the effects of different members of the MAPK pathway on Ets and Smad activity in these cells awaits future investigation.

Collectively these studies establish that CTGF induction by TGF- β in normal fibroblasts is mediated via several discrete promoter elements, comprising a SMAD binding site, which bind a complex comprised of Smad3 and 4 and ETS elements through which members of the Ets family regulate CTGF expression. In turn these transcription factors are regulated by components of the MAPK and PKC signalling pathways which promote a robust induction of CTGF by TGF- β (Figure 7.1).

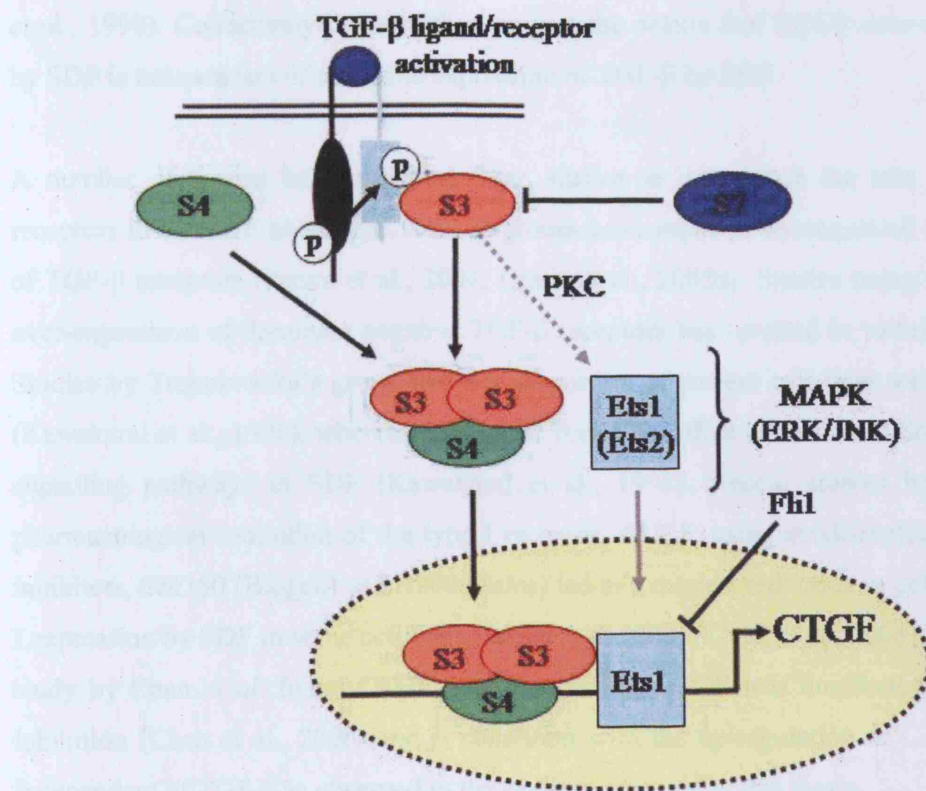


Figure 7.1

A model for the mechanism of CTGF induction by TGF- β in normal dermal fibroblasts.

Data presented in chapter 3 supports the notion of a functional Smad binding site in the proximal promoter of CTGF binding a Smad complex containing Smad3 and Smad4 (Holmes et al., 2003). Subsequent studies have demonstrated CTGF induction by TGF- β to require Ets binding sites, which are able to bind several Ets family members, including Ets1, in a PKC dependent manner (Nakerakanti et al., 2006; Van Beek et al., 2006). In addition members of the MAPK signalling pathway have been implicated in the regulation of CTGF induction by TGF- β . Previous studies have demonstrated MAPK to functionally affect both Smad and Ets transcription factor function.

7.2 Mechanisms of CTGF over-expression in SSc dermal fibroblasts

7.2.1 The role of TGF- β in CTGF over-expression in SSc dermal fibroblasts

Although TGF- β was first postulated in the early 1990s as a mediator of SSc fibrosis (Smith and LeRoy, 1990), subsequent studies have been contradictory as to its precise role in SSc. To determine if dysregulated expression of TGF- β contributed to over-expression of CTGF in SDF, the effects of TGF- β neutralizing antibodies were investigated in Chapter 4. Incubation of SDF with a pan TGF- β neutralizing antibody failed to inhibit over-expression of CTGF by these cells. In addition, over-expression of collagen type I, PAI-1, and the signalling pathways, JNK and ERK1/2, constitutively activated in SDF (Chapter 6; Figure 6.6), were unaffected. Consistent with CTGF over-expression in SDF being independent of autocrine production of TGF- β , secreted levels of bioactive TGF- β were not elevated, in agreement with previous findings (Needleman et al., 1990). Collectively these studies support the notion that CTGF over-expression by SDF is independent of autocrine expression of TGF- β by SDF.

A number of groups have extended these studies to investigate the role of TGF- β receptors in the SDF phenotype. Several groups have reported dysregulated expression of TGF- β receptors (Pannu et al., 2004; Leask et al., 2002a). Studies using adenoviral over-expression of dominant negative TGF- β receptors has resulted in variable effects. Studies by Trojanowska's group found a proportion of patient cell lines were affected (Kawakami et al., 1998), whereas Mori *et al.* found no effect on activated down stream signalling pathways in SDF (Kawakami et al., 1998). Recent studies have shown pharmacological inhibition of the type I receptor, ALK5, using small-molecule kinase inhibitors, SM350 (Biogen) or SD208 (Scios) led to a modest reduction in collagen type I expression by SDF in some cell lines (Ishida et al., 2006; Chen et al., 2006). This latter study by Chen *et al.* found CTGF over-expression in SDF was unaffected by ALK5 inhibition (Chen et al., 2006) and is consistent with the up-regulation of CTGF being independent of TGF- β as observed in the studies presented in this thesis.

7.2.2 Role of Smads in CTGF over-expression

The studies presented here have specifically examined the regulation of CTGF over-expression in SDF. Gene expression studies have found CTGF to be one of the most consistently, differentially expressed genes in SSc. Many of the gene transcripts over-expressed in SDF, like CTGF, are TGF- β responsive. However a notable feature of these gene profiling studies has been the absence of elevated expression of TGF- β

transcripts (Shi-wen et al., 2000; Gardner et al., 2006; Whitfield et al., 2003; Zhou et al., 2001), and consistent with the TGF- β independent over-expression of CTGF observed in these studies Zhou *et al.* recently suggested that SDF maintain a cellular “memory” of TGF- β (Zhou et al., 2005b), however the underlying mechanism remains unknown. Given the requirement of Smads in the regulation of CTGF by TGF- β in normal fibroblasts, it was anticipated that CTGF over-expression in SDF may result from dysregulated Smad activation.

Two approaches were used to address Smad activity in SDF, and its role in CTGF over-expression: transient transfection of a CTGF promoter construct with point mutations in the SMAD binding site, in conjunction with the Smad3 promoter reporter construct CAGA, and Western blotting to determine expression of total protein levels of Smads and nuclear levels of Smad3. Both approaches gave similar results, in that Smad activity was unaltered in SDF. Notably total protein levels of Smad 3, 4 and 7 and nuclear levels of Smad3 were similar in SDF compared to healthy control dermal fibroblasts. By contrast to data presented in Chapter 4, a number of groups have noted dysregulated expression of Smads (Asano et al., 2004b; Dong et al., 2002; Mori et al., 2003), although there is little consensus between these three groups regarding which members of the Smad pathway are affected (Chapter 4; 4.5). Interestingly two of these groups demonstrate that over-expression of Smad7 did not affect the elevated expression of two functionally relevant promoters, COL1A2 (Asano et al., 2004b) and PAI-1 (Dong et al., 2002). This is consistent with the inability of Smad7 to repress elevated expression of the CTGF promoter reporter construct (Chapter 4; Figure 4.6). In contrast to these observations, and studies presented here, Mori *et al.* demonstrated the synthetic promoter, containing four multimerised SMAD consensus binding sites, showed a marked reduction in response to over-expression of Smad7 in SDF (Mori et al., 2003).

The reason for the lack of consensus in these reports of Smad expression in SDF is unclear, although it may reflect differences in disease stage, site of biopsy, disease subset or cell culture conditions. In addition an initial criterion for the selection of SDF cell lines in the studies in this thesis was elevated expression of CTGF. Thus increased CTGF expression, although exhibited by the majority of SDF, may reflect a disease stage in which Smad dysregulation is not present. Future studies defining the activation status and expression of Smads in SDF derived from areas of non-fibrotic, inflammatory

and fibrotic skin in relation to CTGF expression will help define the relationship of Smad dysfunction and disease state.

7.2.3 The role of Sp1-like factors in CTGF over-expression

To delineate the mechanism of CTGF over-expression in SDF a series of promoter deletion constructs were used to define the smallest region of the CTGF promoter able to confer elevated promoter activity in SDF. Using this approach, a short region in the CTGF promoter was identified, which contained DNA elements able to confer constitutively higher promoter activity to a reporter construct in SDF.

Taking a similar approach to those employed in Chapter 3, the mechanisms controlling this region of the CTGF promoter formed the basis of studies presented in Chapters 5 and 6. Point mutations and EMSA identified a functional Sp-binding site within this region. Consistent with this site binding Sp1-like/ KLF family members over-expression of the most studied family member, Sp1, markedly induced CTGF promoter activity in normal fibroblasts, whereas Sp3 repressed Sp1 activation of CTGF. This observation is consistent with the opposing effects these Sp-family members have been reported to have on collagen gene expression (Ghayor et al., 2001). To define the relevance of this site and the factors binding it to CTGF over-expression in SDF two approaches were taken; pharmacological inhibition of GC binding proteins using mithramycin; and CTGF promoter reporter constructs containing point mutations in the Sp-binding site transiently transfected into SDF (Chapter 5; Figure 5.7). These approaches confirmed this site and the factors binding this region were important in the over-expression of CTGF in SDF. EMSA of SDF nuclear extract demonstrated increased binding of Sp1-like transcription factors to double stranded oligo-nucleotides to a consensus Sp-binding site and one spanning the endogenous Sp-binding site of the CTGF promoter.

The increased binding of Sp1-like transcription factors in SDF nuclear extracts could be due either to up-regulation of these factors, such that increased amounts are available in the nucleus to bind, or to 'activation' of Sp1-like factors increasing the binding affinity of these factors for the promoter. In Chapter 5 the levels of Sp1, Sp3 and 'activated' Sp1 were investigated (Chapter 5; Figure 5.9). By Western blotting there were no differences in expression of either Sp1 or Sp3 in SDF (Chapter 5; Figure 5.9). By contrast an ELISA based assay found SDF nuclear extracts exhibited a marked increase in Sp1 binding activity. This observation is in keeping with recent reports demonstrating

elevated Sp1 binding activity in SDF (Ihn and Tamaki, 2000; Jinnin et al., 2006). Overall the results are consistent with the notion that increased activated Sp1 binding, in SDF nuclear extracts, modulates the elevated expression of CTGF. Defining the precise factors binding this Sp-site awaits EMSA supershift studies, utilising antibodies against members of the Sp1-like/KLF family of transcription factors. The effects of siRNA for Sp1 and/or Mithramycin on the gene expression profile of SDF will further define the role played by Sp1-like/KLF family members in SSc.

7.2.4 MAPK signalling pathways and SDF phenotype

In view of the marked increase in Sp-binding and elevated levels of Sp1 in SDF nuclear extracts presented here and in an earlier studies, Chapter 6 focused on the mechanism of Sp1 activation. Previous studies have shown phosphorylation and activation of Sp1 to be regulated by the MAPK, ERK1/2 (Tourkina et al., 2005). Profiling the expression of members of the MAPK family establish SDF protein extracts contained elevated phosphorylated JNK1 and ERK1/2 but not p38. Several subsequent reports confirm increased phosphorylation of JNK and ERK activation in SDF whereas the activation status of p38 remains conflicting (Discussed in Chapter 6; 6.5). The studies presented here are the first to date to profile all three MAPK pathways in relation to the expression of a functionally relevant gene, CTGF, in SDF. It remains unclear if the elevated cellular levels of phosphorylated p38, observed by Ihn *et al.*, occur independently of activation of ERK and JNK pathways in these cells (Ihn et al., 2005).

Given the constitutively activated ERK1/2 and JNK pathways in SDF and previous reports implicating ERK1/2 in increased serum induced Sp1 binding the effects of pharmacological inhibitors of both MAPK pathways on serum induced CTGF and a Sp1-like reporter construct were investigated. As expected, inhibition of ERK1/2 repressed serum induction of reporter activity in both constructs, whereas repression of JNK had no discernable effects on serum induction. Given this the effects of ERK1/2 repression on CTGF over-expression was investigated. Pharmacological repression of ERK1/2 in SDF markedly reduced CTGF promoter activity and total protein levels. Consistent with this effect being mediated through Sp-binding, EMSA demonstrated repression of ERK led to a marked reduction in Sp1-like DNA binding to the CTGF promoter. Although these studies presented in Chapter 6 are consistent with ERK1/2 regulating the activation of Sp1 and subsequent activation of the CTGF promoter, it awaits future studies to define the composition of transcription factors binding the Sp-

binding site and the effects of ERK on this complex in the context of both normal and SSc fibroblasts.

Collectively these studies establish that SDF contain elevated phosphorylated JNK1 and ERK1/2. Pharmacological inhibition of ERK represses over-expression of CTGF in SDF via inhibition of Sp1-like binding to critical elements within the proximal promoter of CTGF. Furthermore SDF contain elevated levels of activated Sp1 and support the notion that increased activation of Sp1 are likely to play a role in the dysregulated expression of CTGF by SDF (Figure 7.2).

7.2.5 The Basal Control Element-1 (BCE-1)

In contrast to the original studies by Grotendorst *et al* (Grotendorst et al., 1996), mutation of the previously described novel TGF- β response element, T β RE, did not affect the ability of CTGF promoter reporter constructs to respond to TGF- β . Intriguingly deletion of this site led to a marked reduction in basal CTGF promoter activity and led to this element being termed the BCE-1 (Holmes et al., 2001). The factors binding this element remain unknown. In silico analysis of the BCE-1 region identifies three putative transcriptional binding sites with over 75% identity to known transcription factor DNA binding sites; a Nkx2.5, AP-1 and ARP-1 sites. As previously discussed, ET-1 induces CTGF expression in normal fibroblasts via the BCE-1 region (Chapter 3; 3.5) and has been previously shown to induce Nkx2.5 expression in cardiomyocytes (Patel and Kos, 2005). However Nkx2.5 expression is absent in unstimulated fibroblasts (Ponticos *et al.* personal communication). The effect of ARP-1 on CTGF is unknown, whereas the AP-1 family members c-jun and junB regulate basal expression of CTGF through as yet undefined promoter elements (Holmes *et al.* in preparation). Transient transfection of SDF with a CTGF promoter reporter construct containing a point mutation in the BCE-1 element resulted in a marked reduction in the elevated expression of CTGF promoter activity (Chapter 4; Figure 4.7). It is interesting to note that whereas the expression of Nkx2.5 and ARP-1 in SSc is unknown, a recent study has found elevated AP-1 binding activity in SDF nuclear extracts (Cho et al., 2006). Future studies to define the factor(s) binding the BCE-1 element and the interaction with other factors controlling basal expression of CTGF, such as those binding the Sp-binding site, are essential to understanding the underlying mechanism of CTGF over expression in SDF.

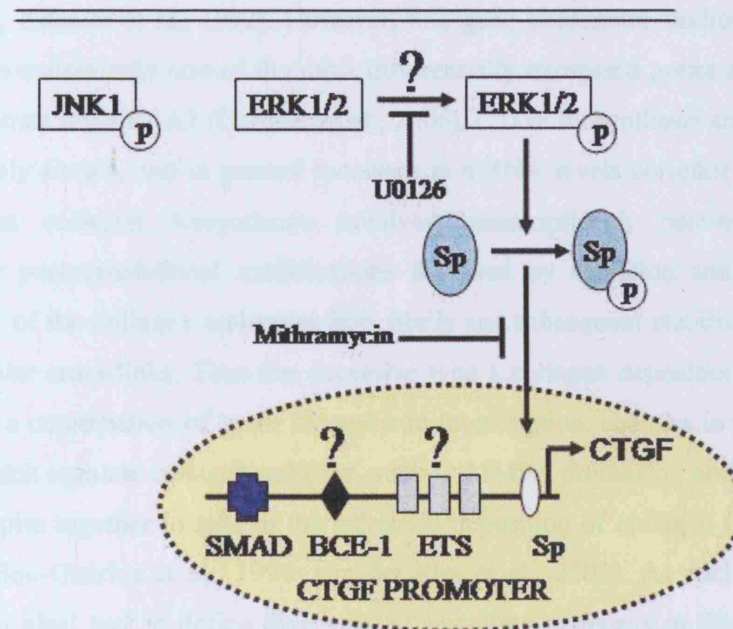


Figure 7.2

Mechanism of CTGF over-expression in SDF

Deletion of the BCE-1 and Sp- DNA binding sites in a CTGF promoter reporter construct leads to a marked reduction in CTGF promoter activity in SDF, although the factors binding these sites remain unknown. In addition SDF have elevated levels of phosphorylated ERK1/2 and JNK1. Repression of ERK leads to a marked repression in Sp binding. In addition repression of ERK or Sp DNA interactions represses CTGF over-expression in SDF.

7.3 Future studies

There are several approaches by which the studies described in this thesis may be expanded and developed towards a better understanding of the regulation of CTGF in SSc and CTGF function in fibrosis.

- Transfections - improved reporter gene delivery (Section 7.3.1)
- Defining the transcription factors binding and interaction of the ETS, BCE-1 and Sp1-like DNA binding sites (Section 7.3.2)
- The function of MAPK in dysregulated CTGF over-expression (Section 7.3.3)
- Transgenic animals in defining CTGF function (Section 7.3.4)

7.3.1 Defining regulatory elements in genes: transfections

To date the most extensively studied functional promoter in context of SSc is COL1A2 (Asano et al., 2004b; Cicchillitti et al., 2004; Ichiki et al., 1997; Ihn et al., 2001b; Jinnin et al., 2006; Kikuchi et al., 1992). However, SSc gene expression studies have shown CTGF to be consistently one of the most differentially expressed genes *in vivo* and *in vitro* in contrast to COL1A2 (Gardner et al., 2006). CTGF biosynthesis and secretion is comparatively simple, and in general increases in mRNA levels correlate with protein. By contrast collagen biosynthesis involves transcriptional, post-transcriptional, intracellular post-translational modifications followed by secretion and extracellular aggregation of the collagen molecules into fibrils and subsequent stabilisation through intermolecular cross-links. Thus the excessive type I collagen deposition *in vivo* may result from a combination of small increases in transcription, changes in expression of proteins which regulate collagen turnover, such as MMPs, processing and cross linking which conspire together to lead to the increased deposition of collagen (McWhirter et al., 1994; Bou-Gharios et al., 1994; van der Slot et al., 2003). As such, CTGF may represent an ideal tool to define dysregulated signalling pathways in SSc and explore their direct effects on this functionally relevant promoter.

The data presented in this thesis have relied extensively on a promoter based approach to assay dysregulation in CTGF expression in SDF. The euphemism ‘promoter bashing’ applies to studies of this kind, in which the regulation of genes is examined through linking portions of the non-coding DNA to ‘reporter’ genes, such as β -gal and luciferase. In the 1970s Graham *et al.* found that coating DNA with DEAE-dextran facilitates DNA passing through the cell membrane, and the process was termed ‘transfection’ (Graham and van der Eb, 1973). Subsequently, transfection products containing lipid and non-liposomal reagents, used in the course of the studies presented in this thesis, have resulted in increased transfection efficiencies. In the studies presented within this thesis, transfection efficiencies of primary dermal fibroblasts were relatively low, consistent with experiences within this and other laboratories (Chapter 3; 3.3.2.3). Although only a small proportion of the cells was transfected, the activity of reporter and deletion promoter constructs mirrored studies to determine the expression and activation status of endogenous proteins. This supports the use of transient transfections to define promoter elements as a valid methodology that reflects the endogenous state of the cells examined, despite low transfection efficiencies.

The use of improved gene delivery technologies will be essential in future studies outlined in 7.2.2; 7.2.3; 7.2.4. Although electroporation of mammalian cells results in a high proportion of cells being transfected, it remains impractical, given the large number of cells required due to the limited availability and passage of patient derived cell lines. Thus viral based systems, such as adeno- or lenti- viral, are the most attractive having several advantages over the use of lipofectamine based systems (Pannu et al., 2004): (1) Both viral systems result in a high level of transfection efficiency, which would allow for the disruption of specific signalling pathways and the effects on endogenous protein levels to be determined (2) Unlike adeno-, lenti-viruses integrate DNA into the host genome and can infect non-dividing cells. Given the role of chromatin in the regulation of gene expression lenti-viral delivery systems the future use of lenti-viral delivered promoter reporter constructs and dominant negative and constitutively active components of signalling pathways to define the regulation of CTGF in a disease context will prove highly advantageous.

7.3.2 CTGF over-expression in SDF: Interaction of the ETS, SMAD, BCE-1 and Sp1-like DNA binding sites

Given the apparent 'memory' of TGF- β and the Smad independent over-expression of CTGF, it remains to define the role of the recently identified ETS binding sites in CTGF over-expression in SDF. Given Fli1, which inhibits CTGF expression, is down regulated in SDF (Kubo et al., 2003), it is likely these ETS binding sites and the transcription factors binding them are likely to be important in the regulation of CTGF in the disease context. Viral based transfections of SDF with CTGF promoter reporter constructs with point mutations in the three ETS binding sites, separately and in combination will better define the relevance of these sites to CTGF over-expression in SSc. In combination, EMSA supershift studies to define the factors binding these elements and Western blot analysis to determine the level of expression in SDF will define the relevance of Ets transcription factors in CTGF over-expression.

Like the transcription factors binding ETS sites, the factors binding the Sp1-like and BCE-1 sites also remain unknown. Although data presented here support Sp1 as a prime candidate in binding to the Sp1-like site, and *in silico* analysis supports the notion that AP-1 family members are likely transcription factors to bind the BCE-1 region, it remains for the factors binding these elements to be defined. Using a similar approach to those employed in defining the Ets transcription factors, future studies are needed to

confirm the transcription factors binding the CTGF promoter elements in normal dermal fibroblasts and the protein levels and 'activation' state of these transcription factors in SSc fibroblasts

Studies by Trojanowska's group have shown ETS to act in concert with other transcription factors, notably Sp1 and Smads (Czuwara-Ladykowska et al., 2002; Jinnin et al., 2004; Czuwara-Ladykowska et al., 2001a). Future studies to define the interdependence of these factors using CTGF promoter reporter constructs containing mutations in different combinations of these sites, using viral delivery, are likely to identify the relevant transcription factors important in CTGF over-expression in SDF.

7.3.3 Function of MAPK in dysregulated CTGF over-expression

To date several members of the MAPK family (ERK1/2, JNK1, p38) have been implicated in the normal expression of CTGF and have been reported as dysregulated in SSc. In addition other members of the MAPK family may play a role in the SDF phenotype. For example ERK5 has recently been shown to be equally sensitive to pharmacological inhibitors of ERK1/2 (Nishimoto and Nishida, 2006), although the functional relevance to CTGF and expression in SSc remains unknown. Over-expression of CTGF is also observed in uninvolved, non-fibrotic dermal fibroblasts, although to a lesser degree (Shi-wen et al., 2000). In studies presented here we have shown ERK1/2 to be important in the regulation of CTGF over-expression in SDF. Future studies using a combination of immuno-fluorescence and Western blot analysis to define the activation state and levels of these MAPKs in cell lines and biopsies from uninvolved, involved and leading edge SSc skin will further define the relationship of these signalling pathways with disease stage and progression. In addition, the effects of pharmacological inhibition of the relevant up-regulated MAPK pathways on the key transcription factors, such as the transcription factors binding the Sp-binding site, BCE-1 element, and the direct effects on endogenous CTGF expression, will further define the role of MAPK in disease progression and development. Recent studies have demonstrated constitutive JNK activation in SSc fibrotic lung fibroblasts (Shi-wen et al., 2006a). Given the apparent cell specific requirement of different members of the MAPK for CTGF induction by TGF- β , comparing the expression and 'activation' state of members of the MAPK family in fibroblasts derived from other organs, such as the lung, in combination with the effects of pharmacological inhibition of the relevant up-

regulated MAPK pathways, in particular JNK, on endogenous CTGF expression, will define if common disease mechanisms occur in these different organs.

Intriguingly, studies in Chapter 4 demonstrated condition media from SDF was able to induce PAI-1 activity in the presence of neutralizing TGF- β antibodies (Chapter 4; Figure 4.4). Supporting the notion of a secretable SDF factor, Zhou *et al.* demonstrated fibroblast conditioned media from SSc patients or their mono-zygotic twin induce gene expression in normal fibroblasts of COL1A2, SPARC, and CTGF, typically seen in SSc fibroblasts (Zhou et al., 2005b). Recently Zhou *et al.* has expanded this study and demonstrated that repression of the matricellular protein, SPARC, partially inhibited COL1A2, COL3A1, and CTGF. Intriguingly SPARC has also been implicated in activation of JNK by TGF- β (Francki et al., 2004), whereas CTGF can induce ERK activation (Chen et al., 2004). Determining the effect of CTGF repression alone and in combination with repression of SPARC on fibrogenic gene expression and the activation of the MAPK pathways will shed light on the general role of matricellular proteins in SSc.

Studies from Varga's group demonstrate a proportion of SDF exhibit increased nuclear Smad staining suggesting a sub-set of these cells are activated (Mori et al., 2003). The proposed use of immuno-fluorescence to define the signalling pathways and transcription factors, which regulate these key promoter elements in CTGF, will further assist in defining if SDF dysfunction and over-expression of fibrogenic genes results from an activation of a discrete subset of cells or a generalised activation of all SSc fibroblasts.

7.3.4 CTGF over-expression fibrosis: epiphenomenon or modulator of fibrosis?

CTGF was originally identified over 15 years ago. However the biological function of this protein still remains the focus of intense investigation and debate. It has been suggested the high cysteine content of the CCN proteins has hindered the production of a pure functional recombinant CTGF protein, and thus defining precise mode of action of CTGF has proved problematic (Lau and Lam, 1999; Frazier et al., 1996). Nonetheless, a growing number of reports suggest CTGF may act as a matricellular protein in promoting the actions of other proteins (Bornstein and Sage, 2002). Interestingly, studies by Gore-Hyer have shown CTGF to promote excessive collagen deposition by SDF in the presence of insulin but alone, CTGF had little effect (Gore-

Hyer et al., 2003). This study supports the notion that rather than acting as a typical growth factor in directly eliciting pro-fibrotic effects, CTGF may function to 'condition' the local micro-environment, adapting and enhancing cellular responses to pro-fibrotic factors by integrating and potentiating the action of other proteins in addition to acting directly (Leask and Abraham, 2004; Perbal, 2004; Leask et al., 2002b) (Figure 7.3).

Several reports demonstrate CTGF is over-expressed in uninvolved skin of SSc patients (Shi-wen et al., 2000), and support the notion that CTGF is not in itself sufficient to induce fibrosis (Frazier et al., 1996; Gore-Hyer et al., 2003; Zhou et al., 2005b) and thus may 'prime' the fibroblasts to respond aberrantly to other pro-fibrogenic factors or the ECM environment. Intriguingly CTGF appears essential for many of the biological effects of TGF- β (Xie et al., 2005; Wang et al., 2004) and a noteworthy study by Abreu *et al.* demonstrates CTGF enhances the downstream activities of TGF- β in *xenopus* (Abreu et al., 2002), although the relevance of this latter study on the activation of TGF- β signalling pathways in mammalian cells awaits future studies. Delineating the functional role of CTGF, be it direct, adaptive or both, remains a key unanswered question for the future. Recently two over-expression CTGF transgenic models were reported at the 9th International Workshop on Scleroderma Research (Boston 2006). Both animal models developed fibrosis, although the phenotype of these animals differed, presumably due to the different targeting vectors used to over-expressing CTGF. In addition, the generation of conditional knockouts of CTGF, reported at the same meeting, are likely to be instrumental in defining the role and function of this growth factor/matricellular protein in pathologies such as fibrosis.

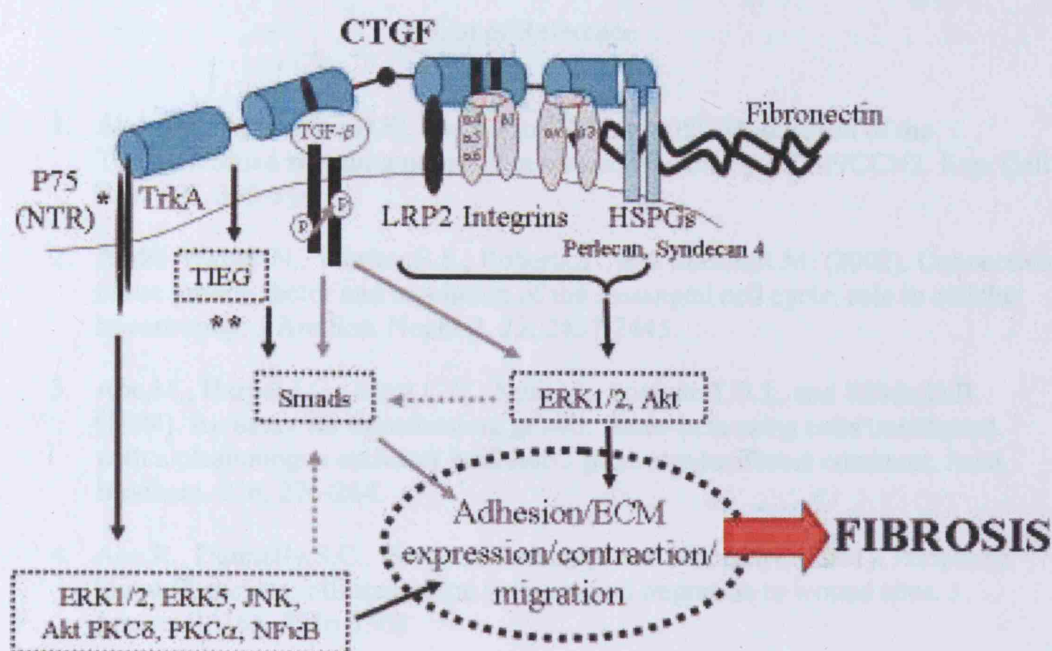


Figure 7.3

Putative model of CTGF function in promoting fibrosis.

The pathological over-expression of CTGF conditions the microenvironment enhancing and prolonging the action of growth factors, such as TGF-β, which are locally elevated in the early stages of tissue injury and inflammation. This leads indirectly to the perpetuation of the pro-fibrotic response, including increased ECM deposition. In addition CTGF directly promotes cell migration and adhesion of mesenchymal cells via integrins and other cellular receptors further promoting fibrosis. * The precise mechanism of interaction of CCN2 with TrkA and p75 remain unknown. ** Direct receptor mediated mechanism of CTGF induction of TIEG remains unknown.

List of Reference

1. Abdel, W.N., Weston, B.S., and Mason, R.M. (2005). Modulation of the TGFbeta/Smad signaling pathway in mesangial cells by CTGF/CCN2. *Exp. Cell Res.* 307, 305-314.
2. Abdel-Wahab, N., Weston, B.S., Roberts, T., and Mason, R.M. (2002). Connective tissue growth factor and regulation of the mesangial cell cycle: role in cellular hypertrophy. *J Am Soc. Nephrol.* 13, 2437-2445.
3. Abe, M., Harpel, J.G., Metz, C.N., Nunes, I., Loskutoff, D.J., and Rifkin, D.B. (1994). An assay for transforming growth factor-beta using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. *Anal. Biochem.* 216, 276-284.
4. Abe, R., Donnelly, S.C., Peng, T., Bucala, R., and Metz, C.N. (2001). Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. *J. Immunol.* 166, 7556-7562.
5. Abecassis, L., Rogier, E., Vazquez, A., Atfi, A., and Bourgeade, M.F. (2004). Evidence for a role of MSK1 in transforming growth factor-beta-mediated responses through p38alpha and Smad signaling pathways. *J Biol. Chem.* 279, 30474-30479.
6. Abou-Shady, M., Friess, H., Zimmermann, A., di Mola, F.F., Guo, X.Z., Baer, H.U., and Buchler, M.W. (2000). Connective tissue growth factor in human liver cirrhosis. *Liver* 20, 296-304.
7. Abraham, D., Lupoli, S., McWhirter, A., Plater-Zyberk, C., Piela, T.H., Korn, J.H., Olsen, I., and Black, C. (1991). Expression and function of surface antigens on scleroderma fibroblasts. *Arthritis Rheum.* 34, 1164-1172.
8. Abraham, D.J., Shiwen, X., Black, C.M., Sa, S., Xu, Y., and Leask, A. (2000). Tumor necrosis factor alpha suppresses the induction of connective tissue growth factor by transforming growth factor-beta in normal and scleroderma fibroblasts. *J. Biol. Chem.* 275, 15220-15225.
9. Abreu, J.G., Ketpura, N.I., Reversade, B., and De Robertis, E.M. (2002). Connective-tissue growth factor (CTGF) modulates cell signalling by BMP and TGF-beta. *Nat. Cell Biol.* 4, 599-604.
10. Agrotis, A., Samuel, M., Prapas, G., and Bobik, A. (1996). Vascular smooth muscle cells express multiple type I receptors for TGF-beta, activin, and bone morphogenetic proteins. *Biochem. Biophys. Res. Commun.* 219, 613-618.
11. Ahmed, S.S. and Tan, F.K. (2003). Identification of novel targets in scleroderma: update on population studies, cDNA arrays, SNP analysis, and mutations. *Curr. Opin. Rheumatol.* 15, 766-771.
12. Akagi, A., Tajima, S., Ishibashi, A., Yamaguchi, N., and Nagai, Y. (1999). Expression of type XVI collagen in human skin fibroblasts: enhanced expression in fibrotic skin diseases. *J. Invest Dermatol.* 113, 246-250.

13. Almendral, J.M., Sommer, D., Macdonald-Bravo, H., Burckhardt, J., Perera, J., and Bravo, R. (1988). Complexity of the early genetic response to growth factors in mouse fibroblasts. *Mol. Cell Biol.* 8, 2140-2148.
14. Andersen, S., van Nieuwenhoven, F.A., Tarnow, L., Rossing, P., Rossing, K., Wieten, L., Goldschmeding, R., and Parving, H.H. (2005). Reduction of urinary connective tissue growth factor by Losartan in type 1 patients with diabetic nephropathy. *Kidney Int.* 67, 2325-2329.
15. Armstrong, S.A., Barry, D.A., Leggett, R.W., and Mueller, C.R. (1997). Casein kinase II-mediated phosphorylation of the C terminus of Sp1 decreases its DNA binding activity. *J. Biol. Chem.* 272, 13489-13495.
16. Arnett, F.C., Cho, M., Chatterjee, S., Aguilar, M.B., Reveille, J.D., and Mayes, M.D. (2001). Familial occurrence frequencies and relative risks for systemic sclerosis (scleroderma) in three United States cohorts. *Arthritis Rheum.* 44, 1359-1362.
17. Arora, K. and Warrior, R. (2001). A new Smurf in the village. *Dev. Cell* 1, 441-442.
18. Artlett, C.M. (2005). Immunology of systemic sclerosis. *Front Biosci.* 10, 1707-1719.
19. Artlett, C.M., Chen, S.J., Varga, J., and Jimenez, S.A. (1998). Modulation of basal expression of the human alpha1(I) procollagen gene (COL1A1) by tandem NF-1/Sp1 promoter elements in normal human dermal fibroblasts. *Matrix Biol.* 17, 425-434.
20. Asano, Y., Ihn, H., Yamane, K., Jinnin, M., Mimura, Y., and Tamaki, K. (2004a). Phosphatidylinositol 3-kinase is involved in alpha2(I) collagen gene expression in normal and scleroderma fibroblasts. *J. Immunol.* 172, 7123-7135.
21. Asano, Y., Ihn, H., Yamane, K., Jinnin, M., Mimura, Y., and Tamaki, K. (2005). Increased Expression of Integrin $\alpha_v\beta_3$ Contributes to the Establishment of Autocrine TGF- β Signaling in Scleroderma Fibroblasts. *J. Immunol.* 175, 7708-7718.
22. Asano, Y., Ihn, H., Yamane, K., Kubo, M., and Tamaki, K. (2004b). Impaired Smad7-Smurf-mediated negative regulation of TGF-beta signaling in scleroderma fibroblasts. *J. Clin. Invest* 113, 253-264.
23. Attisano, L., Carcamo, J., Ventura, F., Weis, F.M., Massague, J., and Wrana, J.L. (1993). Identification of human activin and TGF beta type I receptors that form heteromeric kinase complexes with type II receptors. *Cell* 75, 671-680.
24. Babic, A.M., Chen, C.C., and Lau, L.F. (1999). Fisp12/mouse connective tissue growth factor mediates endothelial cell adhesion and migration through integrin $\alpha_v\beta_3$, promotes endothelial cell survival, and induces angiogenesis in vivo. *Mol. Cell Biol.* 19, 2958-2966.
25. Bai, Y., Yang, C., Hu, K., Elly, C., and Liu, Y.C. (2004). Itch E3 ligase-mediated regulation of TGF-beta signaling by modulating smad2 phosphorylation. *Mol. Cell* 15, 825-831.

26. Barcellos-Hoff, M.H. and Dix, T.A. (1996). Redox-mediated activation of latent transforming growth factor-beta 1. *Mol. Endocrinol.* 10, 1077-1083.
27. Barnett, A.J. (1996). History of Scleroderma. In *Systemic Sclerosis*, Clements P.J and Furst D.E, eds. Williams & Wilkins, Baltimore), pp. 3-19.
28. Battegay, E.J., Raines, E.W., Colbert, T., and Ross, R. (1995). TNF-alpha stimulation of fibroblast proliferation. Dependence on platelet-derived growth factor (PDGF) secretion and alteration of PDGF receptor expression. *J Immunol.* 154, 6040-6047.
29. Belch, J.J. (1991). Raynaud's phenomenon. *Curr. Opin. Rheumatol.* 3, 960-966.
30. Bennett, B.L., Sasaki, D.T., Murray, B.W., O'Leary, E.C., Sakata, S.T., Xu, W., Leisten, J.C., Motiwala, A., Pierce, S., Satoh, Y., Bhagwat, S.S., Manning, A.M., and Anderson, D.W. (2001). SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc. Natl. Acad. Sci. U. S. A* 98, 13681-13686.
31. Bieker, J.J. (2001). Kruppel-like factors: three fingers in many pies. *J. Biol. Chem.* 276, 34355-34358.
32. Birnbaum, M.J., van Wijnen, A.J., Odgren, P.R., Last, T.J., Suske, G., Stein, G.S., and Stein, J.L. (1995). Sp1 trans-activation of cell cycle regulated promoters is selectively repressed by Sp3. *Biochemistry* 34, 16503-16508.
33. Birnboim, H.C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7, 1513-1523.
34. Black, A.R., Jensen, D., Lin, S.Y., and Azizkhan, J.C. (1999). Growth/cell cycle regulation of Sp1 phosphorylation. *J. Biol. Chem.* 274, 1207-1215.
35. Black, C.M. (1995). The aetiopathogenesis of systemic sclerosis: thick skin--thin hypotheses. The Parkes Weber Lecture *J. R. Coll. Physicians Lond* 29, 119-130.
36. Black, C.M. and Denton, C.P. (1998b). Scleroderma and related disorders in adults and children. In *Oxford Textbook of Rheumatology*, Oxford University Press), pp. 1217-1247.
37. Black, C.M. and Denton, C.P. (1998a). Scleroderma and related disorders in adults and children. In *Oxford Textbook of Rheumatology*, P.Maddison, D.A.Isenberg, P.Woo, and D.N.Glass, eds. (Oxford: Oxford University Press), pp. 1217-1247.
38. Black, J.L., Burgess, J.K., and Johnson, P.R. (2003). Airway smooth muscle--its relationship to the extracellular matrix. *Respir. Physiol Neurobiol.* 137, 339-346.
39. Blume, S.W., Snyder, R.C., Ray, R., Thomas, S., Koller, C.A., and Miller, D.M. (1991). Mithramycin inhibits SP1 binding and selectively inhibits transcriptional activity of the dihydrofolate reductase gene in vitro and in vivo. *J. Clin. Invest* 88, 1613-1621.
40. Boehm, J.R., Kutz, S.M., Sage, E.H., Staiano-Coico, L., and Higgins, P.J. (1999). Growth state-dependent regulation of plasminogen activator inhibitor type-1

gene expression during epithelial cell stimulation by serum and transforming growth factor-beta1. *J. Cell Physiol* 181, 96-106.

41. Bonello,M.R. and Khachigian,L.M. (2004). Fibroblast growth factor-2 represses platelet-derived growth factor receptor-alpha (PDGFR-alpha) transcription via ERK1/2-dependent Sp1 phosphorylation and an atypical cis-acting element in the proximal PDGFR-alpha promoter. *J. Biol. Chem.* 279, 2377-2382.
42. Bonner,J.C. (2004). Regulation of PDGF and its receptors in fibrotic diseases. *Cytokine Growth Factor Rev.* 15, 255-273.
43. Bonniaud,P., Margetts,P.J., Kolb,M., Haberberger,T., Kelly,M., Robertson,J., and Gauldie,J. (2003). Adenoviral gene transfer of connective tissue growth factor in the lung induces transient fibrosis. *Am. J. Respir. Crit Care Med.* 168, 770-778.
44. Bonniaud,P., Margetts,P.J., Kolb,M., Schroeder,J.A., Kapoun,A.M., Damm,D., Murphy,A., Chakravarty,S., Dugar,S., Higgins,L., Protter,A.A., and Gauldie,J. (2005). Progressive Transforming Growth Factor {beta} 1-induced Lung Fibrosis Is Blocked by an Orally Active ALK5 Kinase Inhibitor. *Am. J. Respir. Crit Care Med.* 171, 889-898.
45. Bonniaud,P., Martin,G., Margetts,P.J., Ask,K., Robertson,J., Gauldie,J., and Kolb,M. (2004). Connective tissue growth factor is crucial to inducing a profibrotic environment in "fibrosis-resistant" BALB/c mouse lungs. *Am J Respir. Cell Mol. Biol.* 31, 510-516.
46. Borellini,F., Aquino,A., Josephs,S.F., and Glazer,R.I. (1990). Increased expression and DNA-binding activity of transcription factor Sp1 in doxorubicin-resistant HL-60 leukemia cells. *Mol. Cell Biol.* 10, 5541-5547.
47. Bornstein,P. and Sage,E.H. (2002). Matricellular proteins: extracellular modulators of cell function. *Curr. Opin. Cell Biol.* 14, 608-616.
48. Bou-Gharios,G., Osman,J., Black,C., and Olsen,I. (1994). Excess matrix accumulation in scleroderma is caused partly by differential regulation of stromelysin and TIMP-1 synthesis. *Clin. Chim. Acta* 231, 69-78.
49. Bouros,D., Wells,A.U., Nicholson,A.G., Colby,T.V., Polychronopoulos,V., Pantelidis,P., Haslam,P.L., Vassilakis,D.A., Black,C.M., and du Bois,R.M. (2002). Histopathologic subsets of fibrosing alveolitis in patients with systemic sclerosis and their relationship to outcome. *Am J Respir. Crit Care Med.* 165, 1581-1586.
50. Bouwman,P., Gollner,H., Elsasser,H.P., Eckhoff,G., Karis,A., Grosveld,F., Philipsen,S., and Suske,G. (2000). Transcription factor Sp3 is essential for post-natal survival and late tooth development. *EMBO J.* 19, 655-661.
51. Bradham,D.M., Igarashi,A., Potter,R.L., and Grotendorst,G.R. (1991). Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. *J. Cell Biol.* 114, 1285-1294.

52. Branton, M.H. and Kopp, J.B. (1999). TGF-beta and fibrosis. *Microbes. Infect.* *1*, 1349-1365.
53. Brew, K., Dinakarpanian, D., and Nagase, H. (2000). Tissue inhibitors of metalloproteinases: evolution, structure and function. *Biochim. Biophys. Acta* *1477*, 267-283.
54. Brigstock, D.R. (1999). The connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family. *Endocr. Rev.* *20*, 189-206.
55. Brigstock, D.R. (2003). The CCN family: a new stimulus package. *J. Endocrinol.* *178*, 169-175.
56. Brigstock, D.R., Goldschmeding, R., Katsube, K.I., Lam, S.C., Lau, L.F., Lyons, K., Naus, C., Perbal, B., Riser, B., Takigawa, M., and Yeger, H. (2003). Proposal for a unified CCN nomenclature. *Mol. Pathol.* *56*, 127-128.
57. Bronstein, I., Fortin, J.J., Voyta, J.C., Juo, R.R., Edwards, B., Olesen, C.E., Lijam, N., and Kricka, L.J. (1994). Chemiluminescent reporter gene assays: sensitive detection of the GUS and SEAP gene products. *Biotechniques* *17*, 172-177.
58. Brunner, A., Chinn, J., Neubauer, M., and Purchio, A.F. (1991). Identification of a gene family regulated by transforming growth factor-beta. *DNA Cell Biol.* *10*, 293-300.
59. Bucala, R., Spiegel, L.A., Chesney, J., Hogan, M., and Cerami, A. (1994). Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. *Mol. Med.* *1*, 71-81.
60. Bunn, C.C., Denton, C.P., Shi-wen, X., Knight, C., and Black, C.M. (1998). Anti-RNA polymerases and other autoantibody specificities in systemic sclerosis. *Br. J Rheumatol.* *37*, 15-20.
61. Cao, Y.X., Ramirez, M.I., and Williams, M.C. (2003). Enhanced binding of Sp1/Sp3 transcription factors mediates the hyperoxia-induced increased expression of the lung type I cell gene T1alpha. *J. Cell Biochem.* *89*, 887-901.
62. Carter, B.D., Kaltschmidt, C., Kaltschmidt, B., Offenhauser, N., Bohm-Matthaei, R., Baeuerle, P.A., and Barde, Y.A. (1996). Selective activation of NF-kappa B by nerve growth factor through the neurotrophin receptor p75. *Science* *272*, 542-545.
63. Cash, D.E., Gates, P.B., Imokawa, Y., and Brookes, J.P. (1998). Identification of new connective tissue growth factor as a target of retinoid regulation in limb blastemal cells. *Gene* *222*, 119-124.
64. Chacko, B.M., Qin, B.Y., Tiwari, A., Shi, G., Lam, S., Hayward, L.J., De Caestecker, M., and Lin, K. (2004). Structural basis of heteromeric smad protein assembly in TGF-beta signaling. *Mol. Cell* *15*, 813-823.
65. Chadjichristos, C., Ghayor, C., Kypriotou, M., Martin, G., Renard, E., Ala-Kokko, L., Suske, G., de Crombrughe, B., Pujol, J.P., and Galera, P. (2003). Sp1 and Sp3 transcription factors mediate interleukin-1 beta down-regulation of

- human type II collagen gene expression in articular chondrocytes. *J. Biol. Chem.* 278, 39762-39772.
66. Chambers,R.C., Leoni,P., Blanc-Brude,O.P., Wembridge,D.E., and Laurent,G.J. (2000). Thrombin is a potent inducer of connective tissue growth factor production via proteolytic activation of protease-activated receptor-1. *J. Biol. Chem.* 275, 35584-35591.
 67. Chambers,R.C., Leoni,P., Kaminski,N., Laurent,G.J., and Heller,R.A. (2003). Global expression profiling of fibroblast responses to transforming growth factor-beta1 reveals the induction of inhibitor of differentiation-1 and provides evidence of smooth muscle cell phenotypic switching. *Am. J. Pathol.* 162, 533-546.
 68. Chamboredon,S. and Castellazzi,M. (2005). v-Jun downregulates the alpha 2 (I) collagen target gene indirectly through Sp1/3. *Oncogene* 24, 2547-2557.
 69. Chang,E. and Goldberg,H. (1995). Requirements for transforming growth factor-beta regulation of the pro-alpha 2(I) collagen and plasminogen activator inhibitor-1 promoters. *J Biol. Chem.* 270, 4473-4477.
 70. Chaqour,B., Yang,R., and Sha,Q. (2006). Mechanical stretch modulates the promoter activity of the profibrotic factor CCN2 through increased actin polymerization and NF-kappa B activation. *J. Biol. Chem.*
 71. Cheifetz,S., Bellon,T., Cales,C., Vera,S., Bernabeu,C., Massague,J., and Letarte,M. (1992). Endoglin is a component of the transforming growth factor-beta receptor system in human endothelial cells. *J. Biol. Chem.* 267, 19027-19030.
 72. Cheifetz,S., Weatherbee,J.A., Tsang,M.L., Anderson,J.K., Mole,J.E., Lucas,R., and Massague,J. (1987). The transforming growth factor-beta system, a complex pattern of cross-reactive ligands and receptors. *Cell* 48, 409-415.
 73. Chen,C.C., Chen,N., and Lau,L.F. (2001a). The angiogenic factors Cyr61 and connective tissue growth factor induce adhesive signaling in primary human skin fibroblasts. *J Biol. Chem.* 276, 10443-10452.
 74. Chen,S.J., Yuan,W., Lo,S., Trojanowska,M., and Varga,J. (2000). Interaction of smad3 with a proximal smad-binding element of the human alpha2(I) procollagen gene promoter required for transcriptional activation by TGF-beta. *J. Cell Physiol* 183, 381-392.
 75. Chen,X., Weisberg,E., Fridmacher,V., Watanabe,M., Naco,G., and Whitman,M. (1997). Smad4 and FAST-1 in the assembly of activin-responsive factor. *Nature* 389, 85-89.
 76. Chen,Y., Abraham,D.J., Shi-wen,X., Pearson,J.D., Black,C.M., Lyons,K.M., and Leask,A. (2004). CCN2 (connective tissue growth factor) promotes fibroblast adhesion to fibronectin. *Mol. Biol. Cell* 15, 5635-5646.
 77. Chen,Y., Blom,I.E., Sa,S., Goldschmeding,R., Abraham,D.J., and Leask,A. (2002). CTGF expression in mesangial cells: involvement of SMADs, MAP kinase, and PKC. *Kidney Int.* 62, 1149-1159.

78. Chen, Y., Segarini, P., Raoufi, F., Bradham, D., and Leask, A. (2001b). Connective tissue growth factor is secreted through the Golgi and is degraded in the endosome. *Exp. Cell Res.* 271, 109-117.
79. Chen, Y., Shi-wen, X., Eastwood, M., Black, C.M., Denton, C.P., Leask, A., and Abraham, D.J. (2006). Contribution of activin receptor-like kinase 5 (transforming growth factor beta receptor type I) signaling to the fibrotic phenotype of scleroderma fibroblasts. *Arthritis Rheum.* 54, 1309-1316.
80. Chesney, J., Bacher, M., Bender, A., and Bucala, R. (1997). The peripheral blood fibrocyte is a potent antigen-presenting cell capable of priming naive T cells in situ. *Proc. Natl. Acad. Sci. U. S. A* 94, 6307-6312.
81. Chesney, J. and Bucala, R. (2000). Peripheral blood fibrocytes: mesenchymal precursor cells and the pathogenesis of fibrosis. *Curr. Rheumatol. Rep.* 2, 501-505.
82. Chesney, J., Metz, C., Stavitsky, A.B., Bacher, M., and Bucala, R. (1998). Regulated production of type I collagen and inflammatory cytokines by peripheral blood fibrocytes. *J. Immunol.* 160, 419-425.
83. Chizzolini, C., Raschi, E., Rezzonico, R., Testoni, C., Mallone, R., Gabrielli, A., Facchini, A., Del Papa, N., Borghi, M.O., Dayer, J.M., and Meroni, P.L. (2002). Autoantibodies to fibroblasts induce a proadhesive and proinflammatory fibroblast phenotype in patients with systemic sclerosis. *Arthritis Rheum.* 46, 1602-1613.
84. Cho, J.W., Kim, J.Y., Kim, C.W., and Lee, K.S. (2006). Down-regulation of TGF-beta1-induced type I collagen synthesis by AP-1 transcription factor decoy in scleroderma fibroblasts. *J. Dermatol. Sci.* 43(3), 207-209.
85. Choi, H.S., Hwang, C.K., Kim, C.S., Song, K.Y., Law, P.Y., Wei, L.N., and Loh, H.H. (2005). Transcriptional regulation of mouse mu opioid receptor gene: Sp3 isoforms (M1, M2) function as repressors in neuronal cells to regulate the mu opioid receptor gene. *Mol. Pharmacol.* 67, 1674-1683.
86. Chujo, S., Shirasaki, F., Kawara, S., Inagaki, Y., Kinbara, T., Inaoki, M., Takigawa, M., and Takehara, K. (2005). Connective tissue growth factor causes persistent proalpha2(I) collagen gene expression induced by transforming growth factor-beta in a mouse fibrosis model. *J. Cell Physiol* 203, 447-456.
87. Chun, R.F., Semmes, O.J., Neuveut, C., and Jeang, K.T. (1998). Modulation of Sp1 phosphorylation by human immunodeficiency virus type 1 Tat. *J. Virol.* 72, 2615-2629.
88. Cicchillitti, L., Jimenez, S.A., Sala, A., and Saitta, B. (2004). B-Myb acts as a repressor of human COL1A1 collagen gene expression by interacting with Sp1 and CBF factors in scleroderma fibroblasts. *Biochem. J.* 378, 609-616.
89. Claman, H.N., Giorno, R.C., and Seibold, J.R. (1991). Endothelial and fibroblastic activation in scleroderma. The myth of the "uninvolved skin". *Arthritis Rheum.* 34, 1495-1501.

90. Clark,R.A.F. (1996). Wound Repair: Overview and General Considerations. In *The Molecular and Cellular Biology of Wound Repair*, R.A.F.Clark, ed. (New York: Plenum Press).
91. Coghlan,J.G. and Mukerjee,D. (2001). The heart and pulmonary vasculature in scleroderma: clinical features and pathobiology. *Curr. Opin. Rheumatol.* *13*, 495-499.
92. Cohen,S.N., Chang,A.C., and Hsu,L. (1972). Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. *Proc. Natl. Acad. Sci. U. S. A* *69*, 2110-2114.
93. Coker,R.K., Laurent,G.J., Shahzeidi,S., Lympny,P.A., du Bois,R.M., Jeffery,P.K., and McAnulty,R.J. (1997). Transforming growth factors-beta 1, -beta 2, and -beta 3 stimulate fibroblast procollagen production in vitro but are differentially expressed during bleomycin-induced lung fibrosis. *Am. J. Pathol.* *150*, 981-991.
94. Cook,T., Gebelein,B., Mesa,K., Mladek,A., and Urrutia,R. (1998). Molecular cloning and characterization of TIEG2 reveals a new subfamily of transforming growth factor-beta-inducible Sp1-like zinc finger-encoding genes involved in the regulation of cell growth. *J. Biol. Chem.* *273*, 25929-25936.
95. Courey,A.J., Holtzman,D.A., Jackson,S.P., and Tjian,R. (1989). Synergistic activation by the glutamine-rich domains of human transcription factor Sp1. *Cell* *59*, 827-836.
96. Crean,J.K., Finlay,D., Murphy,M., Moss,C., Godson,C., Martin,F., and Brady,H.R. (2002). The role of p42/44 MAPK and protein kinase B in connective tissue growth factor induced extracellular matrix protein production, cell migration, and actin cytoskeletal rearrangement in human mesangial cells. *J. Biol. Chem.* *277*, 44187-44194.
97. Crean,J.K., Furlong,F., Finlay,D., Mitchell,D., Murphy,M., Conway,B., Brady,H.R., Godson,C., and Martin,F. (2004). Connective tissue growth factor [CTGF]/CCN2 stimulates mesangial cell migration through integrated dissolution of focal adhesion complexes and activation of cell polarization. *FASEB J.* *18*, 1541-1543.
98. Cukierman,E., Pankov,R., Stevens,D.R., and Yamada,K.M. (2001). Taking cell-matrix adhesions to the third dimension. *Science* *294*, 1708-1712.
99. Czuwara-Ladykowska,J., Sementchenko,V.I., Watson,D.K., and Trojanowska,M. (2002). Ets1 is an effector of the transforming growth factor beta (TGF-beta) signaling pathway and an antagonist of the profibrotic effects of TGF-beta. *J. Biol. Chem.* *277*, 20399-20408.
100. Czuwara-Ladykowska,J., Shirasaki,F., Jackers,P., Watson,D.K., and Trojanowska,M. (2001b). Fli-1 inhibits collagen type I production in dermal fibroblasts via an Sp1-dependent pathway. *J. Biol. Chem.* *276*, 20839-20848.
101. Czuwara-Ladykowska,J., Shirasaki,F., Jackers,P., Watson,D.K., and Trojanowska,M. (2001a). Fli-1 inhibits collagen type I production in dermal fibroblasts via an Sp1-dependent pathway. *J Biol. Chem.* *276*, 20839-20848.

102. Dammeier,J., Beer,H.D., Brauchle,M., and Werner,S. (1998a). Dexamethasone is a novel potent inducer of connective tissue growth factor expression. Implications for glucocorticoid therapy. *J. Biol. Chem.* 273, 18185-18190.
103. Dammeier,J., Brauchle,M., Falk,W., Grotendorst,G.R., and Werner,S. (1998b). Connective tissue growth factor: a novel regulator of mucosal repair and fibrosis in inflammatory bowel disease? *Int. J Biochem. Cell Biol.* 30, 909-922.
104. Daniel,C., Wiede,J., Krutzsch,H.C., Ribeiro,S.M., Roberts,D.D., Murphy-Ullrich,J.E., and Hugo,C. (2004). Thrombospondin-1 is a major activator of TGF-beta in fibrotic renal disease in the rat in vivo. *Kidney Int.* 65, 459-468.
105. Daniels,J.T., Schultz,G.S., Blalock,T.D., Garrett,Q., Grotendorst,G.R., Dean,N.M., and Khaw,P.T. (2003). Mediation of transforming growth factor-beta(1)-stimulated matrix contraction by fibroblasts: a role for connective tissue growth factor in contractile scarring. *Am. J. Pathol.* 163, 2043-2052.
106. de Gouville,A.C., Boullay,V., Krysa,G., Pilot,J., Brusq,J.M., Lorient,F., Gauthier,J.M., Papworth,S.A., Laroze,A., Gellibert,F., and Huet,S. (2005). Inhibition of TGF-beta signaling by an ALK5 inhibitor protects rats from dimethylnitrosamine-induced liver fibrosis. *Br. J. Pharmacol.* 145, 166-177.
107. de Graauw,M., Hensbergen,P., and van de,W.B. (2006). Phospho-proteomic analysis of cellular signaling. *Electrophoresis.*
108. Dennig,J., Beato,M., and Suske,G. (1996). An inhibitor domain in Sp3 regulates its glutamine-rich activation domains. *EMBO J.* 15, 5659-5667.
109. Dennler,S., Huet,S., and Gauthier,J.M. (1999). A short amino-acid sequence in MH1 domain is responsible for functional differences between Smad2 and Smad3. *Oncogene* 18, 1643-1648.
110. Dennler,S., Itoh,S., Vivien,D., ten Dijke,P., Huet,S., and Gauthier,J.M. (1998). Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO J.* 17, 3091-3100.
111. Denton,C.P. and Black,C.M. (2005). Targeted therapy comes of age in scleroderma. *Trends Immunol.* 26, 596-602.
112. Denton,C.P. and Black,C.M. (2004). Scleroderma--clinical and pathological advances. *Best. Pract. Res. Clin. Rheumatol.* 18, 271-290.
113. Denton,C.P., Black,C.M., and Abraham,D.J. (2006). Mechanisms and consequences of fibrosis in systemic sclerosis. *Nature Clinical Practice Rheumatology* 3, 134-144.
114. Denton,C.P., Zheng,B., Evans,L.A., Shi-wen,X., Ong,V.H., Fisher,I., Lazaridis,K., Abraham,D.J., Black,C.M., and de Crombrughe,B. (2003). Fibroblast-specific expression of a kinase-deficient type II transforming growth factor beta (TGFbeta) receptor leads to paradoxical activation of TGFbeta signaling pathways with fibrosis in transgenic mice. *J. Biol. Chem.* 278, 25109-25119.

115. Derynck,R. and Zhang,Y.E. (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 425, 577-584.
116. Desmouliere,A., Geinoz,A., Gabbiani,F., and Gabbiani,G. (1993). Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J. Cell Biol.* 122, 103-111.
117. di Mola,F.F., Friess,H., Riesle,E., Koliopanos,A., Buchler,P., Zhu,Z., Brigstock,D.R., Korc,M., and Buchler,M.W. (2002). Connective tissue growth factor is involved in pancreatic repair and tissue remodeling in human and rat acute necrotizing pancreatitis. *Ann. Surg.* 235, 60-67.
118. Dickson,M.C., Martin,J.S., Cousins,F.M., Kulkarni,A.B., Karlsson,S., and Akhurst,R.J. (1995). Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development* 121, 1845-1854.
119. Diot,E., Giraudeau,B., Diot,P., Degenne,D., Ritz,L., Guilmot,J.L., and Lemarie,E. (1999). Is anti-topoisomerase I a serum marker of pulmonary involvement in systemic sclerosis? *Chest* 116, 715-720.
120. Distler,O., Distler,J.H., Scheid,A., Acker,T., Hirth,A., Rethage,J., Michel,B.A., Gay,R.E., Muller-Ladner,U., Matucci-Cerinic,M., Plate,K.H., Gassmann,M., and Gay,S. (2004). Uncontrolled expression of vascular endothelial growth factor and its receptors leads to insufficient skin angiogenesis in patients with systemic sclerosis. *Circ. Res.* 95, 109-116.
121. Distler,O., Pap,T., Kowal-Bielecka,O., Meyringer,R., Guiducci,S., Landthaler,M., Scholmerich,J., Michel,B.A., Gay,R.E., Matucci-Cerinic,M., Gay,S., and Muller-Ladner,U. (2001). Overexpression of monocyte chemoattractant protein 1 in systemic sclerosis: role of platelet-derived growth factor and effects on monocyte chemotaxis and collagen synthesis. *Arthritis Rheum.* 44, 2665-2678.
122. Docagne,F., Gabriel,C., Lebeurrier,N., Lesne,S., Hommet,Y., Plawinski,L., Mackenzie,E.T., and Vivien,D. (2004). Sp1 and Smad transcription factors co-operate to mediate TGF-beta-dependent activation of amyloid-beta precursor protein gene transcription. *Biochem. J.* 383, 393-399.
123. Dodig,T.D., Mack,K.T., Cassarino,D.F., and Clark,S.H. (2001). Development of the tight-skin phenotype in immune-deficient mice. *Arthritis Rheum.* 44, 723-727.
124. Doetzelhofer,A., Rotheneder,H., Lagger,G., Koranda,M., Kurtev,V., Brosch,G., Wintersberger,E., and Seiser,C. (1999). Histone deacetylase 1 can repress transcription by binding to Sp1. *Mol. Cell Biol.* 19, 5504-5511.
125. Dong,C., Li,Z., Alvarez,R., Jr., Feng,X.H., and Goldschmidt-Clermont,P.J. (2000). Microtubule binding to Smads may regulate TGF beta activity. *Mol. Cell* 5, 27-34.
126. Dong,C., Zhu,S., Wang,T., Yoon,W., Li,Z., Alvarez,R.J., ten Dijke,P., White,B., Wigley,F.M., and Goldschmidt-Clermont,P.J. (2002). Deficient Smad7

expression: a putative molecular defect in scleroderma. *Proc. Natl. Acad. Sci. U. S. A* 99, 3908-3913.

127. Duncan,M.R., Frazier,K.S., Abramson,S., Williams,S., Klapper,H., Huang,X., and Grotendorst,G.R. (1999). Connective tissue growth factor mediates transforming growth factor beta-induced collagen synthesis: down-regulation by cAMP. *FASEB J.* 13, 1774-1786.
128. Dynan,W.S. and Tjian,R. (1983a). Isolation of transcription factors that discriminate between different promoters recognized by RNA polymerase II. *Cell* 32, 669-680.
129. Dynan,W.S. and Tjian,R. (1983b). The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. *Cell* 35, 79-87.
130. Dziadzio,M., Smith,R.E., Abraham,D.J., Black,C.M., and Denton,C.P. (2005a). Circulating levels of active transforming growth factor beta1 are reduced in diffuse cutaneous systemic sclerosis and correlate inversely with the modified Rodnan skin score. *Rheumatology (Oxford)* 44, 1518-1524.
131. Dziadzio,M., Usinger,W., Leask,A., Abraham,D., Black,C.M., Denton,C., and Stratton,R. (2005b). N-terminal connective tissue growth factor is a marker of the fibrotic phenotype in scleroderma. *QJM.* 98, 485-492.
132. Dziankowska-Bartkowiak,B., Waszczykowska,E., Zalewska,A., and Sysa-Jedrzejowska,A. (2005). Correlation of endostatin and tissue inhibitor of metalloproteinases 2 (TIMP2) serum levels with cardiovascular involvement in systemic sclerosis patients. *Mediators. Inflamm.* 2005, 144-149.
133. Emili,A., Greenblatt,J., and Ingles,C.J. (1994). Species-specific interaction of the glutamine-rich activation domains of Sp1 with the TATA box-binding protein. *Mol. Cell Biol.* 14, 1582-1593.
134. Engel,M.E., McDonnell,M.A., Law,B.K., and Moses,H.L. (1999). Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription. *J Biol. Chem.* 274, 37413-37420.
135. Fan,W.H., Pech,M., and Karnovsky,M.J. (2000). Connective tissue growth factor (CTGF) stimulates vascular smooth muscle cell growth and migration in vitro. *Eur. J. Cell Biol.* 79, 915-923.
136. Favata,M.F., Horiuchi,K.Y., Manos,E.J., Daulerio,A.J., Stradley,D.A., Feeser,W.S., Van Dyk,D.E., Pitts,W.J., Earl,R.A., Hobbs,F., Copeland,R.A., Magolda,R.L., Scherle,P.A., and Trzaskos,J.M. (1998). Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J. Biol. Chem.* 273, 18623-18632.
137. Felici,A., Wurthner,J.U., Parks,W.T., Giam,L.R., Reiss,M., Karpova,T.S., McNally,J.G., and Roberts,A.B. (2003). TLP, a novel modulator of TGF-beta signaling, has opposite effects on Smad2- and Smad3-dependent signaling. *EMBO J.* 22, 4465-4477.
138. Feng,X.H. and Derynck,R. (2005). Specificity and versatility in tgfbeta signaling through Smads. *Annu. Rev. Cell Dev. Biol.* 21, 659-693.

139. Feng,X.H. and Derynck,R. (1997). A kinase subdomain of transforming growth factor-beta (TGF-beta) type I receptor determines the TGF-beta intracellular signaling specificity. *EMBO J* 16, 3912-3923.
140. Feng,X.H., Lin,X., and Derynck,R. (2000). Smad2, Smad3 and Smad4 cooperate with Sp1 to induce p15(Ink4B) transcription in response to TGF-beta. *EMBO J.* 19, 5178-5193.
141. Ferguson,M.W. and O'Kane,S. (2004). Scar-free healing: from embryonic mechanisms to adult therapeutic intervention. *Philos. Trans. R. Soc. Lond B Biol. Sci.* 359, 839-850.
142. Flanders,K.C., Major,C.D., Arabshahi,A., Aburime,E.E., Okada,M.H., Fujii,M., Blalock,T.D., Schultz,G.S., Sowers,A., Anzano,M.A., Mitchell,J.B., Russo,A., and Roberts,A.B. (2003). Interference with transforming growth factor-beta/Smad3 signaling results in accelerated healing of wounds in previously irradiated skin. *Am J Pathol* 163, 2247-2257.
143. Fleischmajer,R., Gay,S., Meigel,W.N., and Perlish,J.S. (1978). Collagen in the cellular and fibrotic stages of scleroderma. *Arthritis Rheum.* 21, 418-428.
144. Francki,A., McClure,T.D., Brekken,R.A., Motamed,K., Murri,C., Wang,T., and Sage,E.H. (2004). SPARC regulates TGF-beta1-dependent signaling in primary glomerular mesangial cells. *J. Cell Biochem.* 91, 915-925.
145. Frazier,K., Williams,S., Kothapalli,D., Klapper,H., and Grotendorst,G.R. (1996). Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor. *J. Invest Dermatol.* 107, 404-411.
146. Friedrichsen,S., Heuer,H., Christ,S., Cuthill,D., Bauer,K., and Raivich,G. (2005). Gene expression of connective tissue growth factor in adult mouse. *Growth Factors* 23, 43-53.
147. Fu,M., Zhang,J., Zhu,X., Myles,D.E., Willson,T.M., Liu,X., and Chen,Y.E. (2001). Peroxisome proliferator-activated receptor gamma inhibits transforming growth factor beta-induced connective tissue growth factor expression in human aortic smooth muscle cells by interfering with Smad3. *J. Biol. Chem.* 276, 45888-45894.
148. Gabrielli,A., Di Loreto,C., Taborro,R., Candela,M., Sambo,P., Nitti,C., Danieli,M.G., DeLustro,F., Dasch,J.R., and Danieli,G. (1993). Immunohistochemical localization of intracellular and extracellular associated TGF beta in the skin of patients with systemic sclerosis (scleroderma) and primary Raynaud's phenomenon. *Clin. Immunol. Immunopathol.* 68, 340-349.
149. Gan,Y., Shen,Y.H., Wang,J., Wang,X., Utama,B., Wang,J., and Wang,X.L. (2005). Role of histone deacetylation in cell-specific expression of endothelial nitric-oxide synthase. *J. Biol. Chem.* 280, 16467-16475.
150. Gao,R. and Brigstock,D.R. (2005c). A novel integrin {alpha}5{beta}1: binding domain in module 4 of connective tissue growth factor promotes adhesion and migration of activated pancreatic stellate cells. *Gut.*

151. Gao,R. and Brigstock,D.R. (2004). Connective tissue growth factor (CCN2) induces adhesion of rat activated hepatic stellate cells by binding of its C-terminal domain to integrin alpha(v)beta(3) and heparan sulfate proteoglycan. *J. Biol. Chem.* 279, 8848-8855.
152. Gao,R. and Brigstock,D.R. (2005b). Connective tissue growth factor (CCN2) in rat pancreatic stellate cell function: integrin alpha5beta1 as a novel CCN2 receptor. *Gastroenterology* 129, 1019-1030.
153. Gao,R. and Brigstock,D.R. (2005a). Activation of nuclear factor kappa B (NF-kappaB) by connective tissue growth factor (CCN2) is involved in sustaining the survival of primary rat hepatic stellate cells. *Cell Commun. Signal.* 3, 14.
154. Gao,Z., Sasaoka,T., Fujimori,T., Oya,T., Ishii,Y., Sabit,H., Kawaguchi,M., Kurotaki,Y., Naito,M., Wada,T., Ishizawa,S., Kobayashi,M., Nabeshima,Y., and Sasahara,M. (2005). Deletion of the PDGFR-beta gene affects key fibroblast functions important for wound healing. *J. Biol. Chem.* 280, 9375-9389.
155. Gardner,H., Shearstone,J.R., Bandaru,R., Crowell,T., Lynes,M., Trojanowska,M., Pannu,J., Smith,E., Jablonska,S., Blaszczyk,M., Tan,F.K., and Mayes,M.D. (2006). Gene profiling of scleroderma skin reveals robust signatures of disease that are imperfectly reflected in the transcript profiles of explanted fibroblasts. *Arthritis Rheum.* 54, 1961-1973.
156. Garrett,Q., Khaw,P.T., Blalock,T.D., Schultz,G.S., Grotendorst,G.R., and Daniels,J.T. (2004). Involvement of CTGF in TGF-beta1-stimulation of myofibroblast differentiation and collagen matrix contraction in the presence of mechanical stress. *Invest Ophthalmol. Vis. Sci.* 45, 1109-1116.
157. Gartel,A.L. and Shchors,K. (2003). Mechanisms of c-myc-mediated transcriptional repression of growth arrest genes. *Exp. Cell Res.* 283, 17-21.
158. Gay,S., Jones,R.E., Jr., Huang,G.Q., and Gay,R.E. (1989). Immunohistologic demonstration of platelet-derived growth factor (PDGF) and sis-oncogene expression in scleroderma. *J. Invest Dermatol.* 92, 301-303.
159. Gershwin,M.E., Abplanalp,H., Castles,J.J., Ikeda,R.M., van der Water J., Eklund,J., and Haynes,D. (1981). Characterization of a spontaneous disease of white leghorn chickens resembling progressive systemic sclerosis (scleroderma). *J. Exp. Med.* 153, 1640-1659.
160. Ghayor,C., Chadjichristos,C., Herrouin,J.F., Ala-Kokko,L., Suske,G., Pujol,J.P., and Galera,P. (2001). Sp3 represses the Sp1-mediated transactivation of the human COL2A1 gene in primary and de-differentiated chondrocytes. *J. Biol. Chem.* 276, 36881-36895.
161. Gilchrist,F.C., Bunn,C., Foley,P.J., Lympny,P.A., Black,C.M., Welsh,K.I., and du Bois,R.M. (2001). Class II HLA associations with autoantibodies in scleroderma: a highly significant role for HLA-DP. *Genes Immun.* 2, 76-81.
162. Gill,G., Pascal,E., Tseng,Z.H., and Tjian,R. (1994). A glutamine-rich hydrophobic patch in transcription factor Sp1 contacts the dTAFII110 component of the Drosophila TFIID complex and mediates transcriptional activation. *Proc. Natl. Acad. Sci. U. S. A* 91, 192-196.

163. Gore-Hyer,E., Pannu,J., Smith,E.A., Grotendorst,G., and Trojanowska,M. (2003). Selective stimulation of collagen synthesis in the presence of costimulatory insulin signaling by connective tissue growth factor in scleroderma fibroblasts. *Arthritis Rheum.* 48, 798-806.
164. Graham,F.L. and van der Eb,A.J. (1973). Transformation of rat cells by DNA of human adenovirus 5. *Virology* 54, 536-539.
165. Green,M.C., Sweet,H.O., and Bunker,L.E. (1976). Tight-skin, a new mutation of the mouse causing excessive growth of connective tissue and skeleton. *Am. J. Pathol.* 82, 493-512.
166. Greenwel,P., Inagaki,Y., Hu,W., Walsh,M., and Ramirez,F. (1997). Sp1 is required for the early response of alpha2(I) collagen to transforming growth factor-beta1. *J. Biol. Chem.* 272, 19738-19745.
167. Groskreutz DJ (2005). Increased expression and convenience with the new pGL3 luciferase reporter vectors. *Promega Notes* 50, 2.
168. Grotendorst,G.R. and Duncan,M.R. (2005). Individual domains of connective tissue growth factor regulate fibroblast proliferation and myofibroblast differentiation. *FASEB J* 19, 729-738.
169. Grotendorst,G.R., Okochi,H., and Hayashi,N. (1996). A novel transforming growth factor beta response element controls the expression of the connective tissue growth factor gene. *Cell Growth Differ.* 7, 469-480.
170. Gruschwitz,M., Muller,P.U., Sepp,N., Hofer,E., Fontana,A., and Wick,G. (1990). Transcription and expression of transforming growth factor type beta in the skin of progressive systemic sclerosis: a mediator of fibrosis? *J. Invest Dermatol.* 94, 197-203.
171. Gruschwitz,M., von den,D.P., Kellner,I., Hornstein,O.P., and Sterry,W. (1992). Expression of adhesion proteins involved in cell-cell and cell-matrix interactions in the skin of patients with progressive systemic sclerosis. *J. Am. Acad. Dermatol.* 27, 169-177.
172. Gupta,R., Emili,A., Pan,G., Xiao,H., Shales,M., Greenblatt,J., and Ingles,C.J. (1996). Characterization of the interaction between the acidic activation domain of VP16 and the RNA polymerase II initiation factor TFIIB. *Nucleic Acids Res.* 24, 2324-2330.
173. Hagen,G., Muller,S., Beato,M., and Suske,G. (1994). Sp1-mediated transcriptional activation is repressed by Sp3. *EMBO J.* 13, 3843-3851.
174. Haidweiger,E., Novy,M., and Rotheneder,H. (2001). Modulation of Sp1 activity by a cyclin A/CDK complex. *J. Mol. Biol.* 306, 201-212.
175. Han,I. and Kudlow,J.E. (1997). Reduced O glycosylation of Sp1 is associated with increased proteasome susceptibility. *Mol. Cell Biol.* 17, 2550-2558.
176. Harrison,N.K., Cambrey,A.D., Myers,A.R., Southcott,A.M., Black,C.M., du Bois,R.M., Laurent,G.J., and McAnulty,R.J. (1994). Insulin-like growth factor-I

is partially responsible for fibroblast proliferation induced by bronchoalveolar lavage fluid from patients with systemic sclerosis. *Clin. Sci. (Lond)* 86, 141-148.

177. Hashimoto,G., Inoki,I., Fujii,Y., Aoki,T., Ikeda,E., and Okada,Y. (2002). Matrix metalloproteinases cleave connective tissue growth factor and reactivate angiogenic activity of vascular endothelial growth factor 165. *J. Biol. Chem.* 277, 36288-36295.
178. Hashimoto,N., Jin,H., Liu,T., Chensue,S.W., and Phan,S.H. (2004). Bone marrow-derived progenitor cells in pulmonary fibrosis. *J. Clin. Invest* 113, 243-252.
179. Hassel,S., Schmitt,S., Hartung,A., Roth,M., Nohe,A., Petersen,N., Ehrlich,M., Henis,Y.I., Sebald,W., and Knaus,P. (2003). Initiation of Smad-dependent and Smad-independent signaling via distinct BMP-receptor complexes. *J. Bone Joint Surg. Am.* 85-A Suppl 3, 44-51.
180. Hata,A., Lagna,G., Massague,J., and Hemmati-Brivanlou,A. (1998). Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. *Genes Dev.* 12, 186-197.
181. Hata,A., Lo,R.S., Wotton,D., Lagna,G., and Massague,J. (1997). Mutations increasing autoinhibition inactivate tumour suppressors Smad2 and Smad4. *Nature* 388, 82-87.
182. Hayashi,H., Abdollah,S., Qiu,Y., Cai,J., Xu,Y.Y., Grinnell,B.W., Richardson,M.A., Topper,J.N., Gimbrone,M.A., Jr., Wrana,J.L., and Falb,D. (1997). The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. *Cell* 89, 1165-1173.
183. Hayashida,T., Decaestecker,M., and Schnaper,H.W. (2003). Cross-talk between ERK MAP kinase and Smad signaling pathways enhances TGF-beta-dependent responses in human mesangial cells. *FASEB J.* 17, 1576-1578.
184. Helman, Cotton SA, Jayson MIV, Hoyland J, Herrick,A., and Freemont AJ (2005). Microvascular abnormality and TGF-beta in systemic sclerosis. *Rheumatology* 36(Suppl), 30(54A).
185. Heng,E.C., Huang,Y., Black,S.A., Jr., and Trackman,P.C. (2006). CCN2, connective tissue growth factor, stimulates collagen deposition by gingival fibroblasts via module 3 and alpha6- and beta1 integrins. *J. Cell Biochem.* 98, 409-420.
186. Herrick,A.L. and Matucci,C.M. (2001). The emerging problem of oxidative stress and the role of antioxidants in systemic sclerosis. *Clin. Exp. Rheumatol.* 19, 4-8.
187. Hesselstrand,R., Westergren-Thorsson,G., Scheja,A., Wildt,M., and Akesson,A. (2002). The association between changes in skin echogenicity and the fibroblast production of biglycan and versican in systemic sclerosis. *Clin. Exp. Rheumatol.* 20, 301-308.

188. Higgins,D.F., Biju,M.P., Akai,Y., Wutz,A., Johnson,R.S., and Haase,V.H. (2004). Hypoxic induction of Ctgf is directly mediated by Hif-1. *Am. J. Physiol Renal Physiol* 287, F1223-F1232.
189. Higgins,D.F., Lappin,D.W., Kieran,N.E., Anders,H.J., Watson,R.W., Strutz,F., Schlondorff,D., Haase,V.H., Fitzpatrick,J.M., Godson,C., and Brady,H.R. (2003). DNA oligonucleotide microarray technology identifies fisp-12 among other potential fibrogenic genes following murine unilateral ureteral obstruction (UUO): modulation during epithelial-mesenchymal transition. *Kidney Int.* 64, 2079-2091.
190. Hilton,T.L., Li,Y., Dunphy,E.L., and Wang,E.H. (2005). TAF1 histone acetyltransferase activity in Sp1 activation of the cyclin D1 promoter. *Mol. Cell Biol.* 25, 4321-4332.
191. Hishikawa,K., Nakaki,T., and Fujii,T. (2000). Connective tissue growth factor induces apoptosis via caspase 3 in cultured human aortic smooth muscle cells. *Eur. J. Pharmacol.* 392, 19-22.
192. Hishikawa,K., Oemar,B.S., Tanner,F.C., Nakaki,T., Luscher,T.F., and Fujii,T. (1999). Connective tissue growth factor induces apoptosis in human breast cancer cell line MCF-7. *J Biol. Chem.* 274, 37461-37466.
193. Hitraya,E.G., Varga,J., Artlett,C.M., and Jimenez,S.A. (1998). Identification of elements in the promoter region of the alpha1(I) procollagen gene involved in its up-regulated expression in systemic sclerosis. *Arthritis Rheum.* 41, 2048-2058.
194. Ho,K.T. and Reveille,J.D. (2003). The clinical relevance of autoantibodies in scleroderma. *Arthritis Res. Ther.* 5, 80-93.
195. Ho,M., Veale,D., Eastmond,C., Nuki,G., and Belch,J. (2000). Macrovascular disease and systemic sclerosis. *Ann. Rheum. Dis.* 59, 39-43.
196. Hocevar,B.A., Brown,T.L., and Howe,P.H. (1999). TGF-beta induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway. *EMBO J.* 18, 1345-1356.
197. Holmes,A., Abraham,D.J., Chen,Y., Denton,C., Shi-wen,X., Black,C.M., and Leask,A. (2003). Constitutive connective tissue growth factor expression in scleroderma fibroblasts is dependent on Sp1. *J. Biol. Chem.* 278, 41728-41733.
198. Holmes,A., Abraham,D.J., Sa,S., Shiwen,X., Black,C.M., and Leask,A. (2001). CTGF and SMADs, maintenance of scleroderma phenotype is independent of SMAD signaling. *J. Biol. Chem.* 276, 10594-10601.
199. Hoshijima,M., Hattori,T., Inoue,M., Araki,D., Hanagata,H., Miyauchi,A., and Takigawa,M. (2006). CT domain of CCN2/CTGF directly interacts with fibronectin and enhances cell adhesion of chondrocytes through integrin alpha5beta1. *FEBS Lett.* 580, 1376-1382.
200. Hu,B., Wu,Z., and Phan,S.H. (2003). Smad3 mediates transforming growth factor-beta-induced alpha-smooth muscle actin expression. *Am. J. Respir. Cell Mol. Biol.* 29, 397-404.

201. Huang,W., Zhao,S., Ammanamanchi,S., Brattain,M., Venkatasubbarao,K., and Freeman,J.W. (2005). Trichostatin A induces transforming growth factor beta type II receptor promoter activity and acetylation of Sp1 by recruitment of PCAF/p300 to a Sp1.NF-Y complex. *J. Biol. Chem.* 280, 10047-10054.
202. Huber,M.A., Kraut,N., and Beug,H. (2005). Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr. Opin. Cell Biol.* 17, 548-558.
203. Hurvitz,J.R., Suwairi,W.M., Van Hul,W., El Shanti,H., Superti-Furga,A., Roudier,J., Holderbaum,D., Pauli,R.M., Herd,J.K., Van Hul,E.V., Rezai-Delui,H., Legius,E., Le Merrer,M., Al Alami,J., Bahabri,S.A., and Warman,M.L. (1999). Mutations in the CCN gene family member WISP3 cause progressive pseudorheumatoid dysplasia. *Nat. Genet.* 23, 94-98.
204. Ichiki,Y., Smith,E.A., LeRoy,E.C., and Trojanowska,M. (1997). Basic fibroblast growth factor inhibits basal and transforming growth factor-beta induced collagen alpha 2(I) gene expression in scleroderma and normal fibroblasts. *J. Rheumatol.* 24, 90-95.
205. Igarashi,A., Nashiro,K., Kikuchi,K., Sato,S., Ihn,H., Fujimoto,M., Grotendorst,G.R., and Takehara,K. (1996). Connective tissue growth factor gene expression in tissue sections from localized scleroderma, keloid, and other fibrotic skin disorders. *J. Invest Dermatol.* 106, 729-733.
206. Igarashi,A., Nashiro,K., Kikuchi,K., Sato,S., Ihn,H., Grotendorst,G.R., and Takehara,K. (1995). Significant correlation between connective tissue growth factor gene expression and skin sclerosis in tissue sections from patients with systemic sclerosis. *J. Invest Dermatol.* 105, 280-284.
207. Igarashi,A., Okochi,H., Bradham,D.M., and Grotendorst,G.R. (1993). Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. *Mol. Biol. Cell* 4, 637-645.
208. Ihn,H. (2005). Scleroderma, fibroblasts, signaling, and excessive extracellular matrix. *Curr. Rheumatol. Rep.* 7, 156-162.
209. Ihn,H., Ihn,Y., and Trojanowska,M. (2001a). Spl phosphorylation induced by serum stimulates the human alpha2(I) collagen gene expression. *J. Invest Dermatol.* 117, 301-308.
210. Ihn,H., Ohnishi,K., Tamaki,T., LeRoy,E.C., and Trojanowska,M. (1996). Transcriptional regulation of the human alpha2(I) collagen gene. Combined action of upstream stimulatory and inhibitory cis-acting elements. *J. Biol. Chem.* 271, 26717-26723.
211. Ihn,H. and Tamaki,K. (2000). Increased phosphorylation of transcription factor Sp1 in scleroderma fibroblasts: association with increased expression of the type I collagen gene. *Arthritis Rheum.* 43, 2240-2247.
212. Ihn,H. and Trojanowska,M. (1997). Sp3 is a transcriptional activator of the human alpha2(I) collagen gene. *Nucleic Acids Res.* 25, 3712-3717.

213. Ihn,H., Yamane,K., Asano,Y., Jinnin,M., and Tamaki,K. (2006). Constitutively phosphorylated Smad3 interacts with Sp1 and p300 in scleroderma fibroblasts. *Rheumatology (Oxford)* 45, 157-165.
214. Ihn,H., Yamane,K., Kubo,M., and Tamaki,K. (2001b). Blockade of endogenous transforming growth factor beta signaling prevents up-regulated collagen synthesis in scleroderma fibroblasts: association with increased expression of transforming growth factor beta receptors. *Arthritis Rheum.* 44, 474-480.
215. Ihn,H., Yamane,K., and Tamaki,K. (2005). Increased phosphorylation and activation of mitogen-activated protein kinase p38 in scleroderma fibroblasts. *J. Invest Dermatol.* 125, 247-255.
216. Imamura,T., Takase,M., Nishihara,A., Oeda,E., Hanai,J., Kawabata,M., and Miyazono,K. (1997). Smad6 inhibits signalling by the TGF-beta superfamily. *Nature* 389, 622-626.
217. Inagaki,Y., Nemoto,T., Nakao,A., Dijke,P., Kobayashi,K., Takehara,K., and Greenwel,P. (2001). Interaction between GC box binding factors and Smad proteins modulates cell lineage-specific alpha 2(I) collagen gene transcription. *J. Biol. Chem.* 276, 16573-16579.
218. Inoki,I., Shiomi,T., Hashimoto,G., Enomoto,H., Nakamura,H., Makino,K., Ikeda,E., Takata,S., Kobayashi,K., and Okada,Y. (2002). Connective tissue growth factor binds vascular endothelial growth factor (VEGF) and inhibits VEGF-induced angiogenesis. *FASEB J* 16, 219-221.
219. Ioannidis,J.P., Vlachoyiannopoulos,P.G., Haidich,A.B., Medsger,T.A., Jr., Lucas,M., Michet,C.J., Kuwana,M., Yasuoka,H., van den,H.F., Te,B.L., van Laar,J.M., Verbeet,N.L., Matucci-Cerinic,M., Georgountzos,A., and Moutsopoulos,H.M. (2005). Mortality in systemic sclerosis: an international meta-analysis of individual patient data. *Am J Med.* 118, 2-10.
220. Ishida,W., Mori,Y., Lakos,G., Sun,L., Shan,F., Bowes,S., Josiah,S., Lee,W.C., Singh,J., Ling,L.E., and Varga,J. (2006). Intracellular tgf-Beta receptor blockade abrogates smad-dependent fibroblast activation in vitro and in vivo. *J. Invest Dermatol.* 126, 1733-1744.
221. Ito,Y., Aten,J., Bende,R.J., Oemar,B.S., Rabelink,T.J., Weening,J.J., and Goldschmeding,R. (1998). Expression of connective tissue growth factor in human renal fibrosis. *Kidney Int.* 53, 853-861.
222. Ivkovic,S., Yoon,B.S., Popoff,S.N., Safadi,F.F., Libuda,D.E., Stephenson,R.C., Daluiski,A., and Lyons,K.M. (2003). Connective tissue growth factor coordinates chondrogenesis and angiogenesis during skeletal development. *Development* 130, 2779-2791.
223. Jackson,S.P., MacDonald,J.J., Lees-Miller,S., and Tjian,R. (1990). GC box binding induces phosphorylation of Sp1 by a DNA-dependent protein kinase. *Cell* 63, 155-165.
224. Jackson,S.P. and Tjian,R. (1988). O-glycosylation of eukaryotic transcription factors: implications for mechanisms of transcriptional regulation. *Cell* 55, 125-133.

225. Jainchill, J.L., Aaronson, S.A., and Todaro, G.J. (1969). Murine sarcoma and leukemia viruses: assay using clonal lines of contact-inhibited mouse cells. *J. Virol.* 4, 549-553.
226. Janknecht, R., Wells, N.J., and Hunter, T. (1998). TGF-beta-stimulated cooperation of smad proteins with the coactivators CBP/p300. *Genes Dev.* 12, 2114-2119.
227. Javelaud, D., Laboureaud, J., Gabison, E., Verrecchia, F., and Mauviel, A. (2003). Disruption of basal JNK activity differentially affects key fibroblast functions important for wound healing. *J. Biol. Chem.* 278, 24624-24628.
228. Javelaud, D. and Mauviel, A. (2005). Crosstalk mechanisms between the mitogen-activated protein kinase pathways and Smad signaling downstream of TGF-beta: implications for carcinogenesis. *Oncogene* 24, 5742-5750.
229. Jedsadayanmata, A., Chen, C.C., Kireeva, M.L., Lau, L.F., and Lam, S.C. (1999). Activation-dependent adhesion of human platelets to Cyr61 and Fisp12/mouse connective tissue growth factor is mediated through integrin alpha(IIb)beta(3). *J. Biol. Chem.* 274, 24321-24327.
230. Jelaska, A. and Korn, J.H. (2000). Role of apoptosis and transforming growth factor beta1 in fibroblast selection and activation in systemic sclerosis. *Arthritis Rheum.* 43, 2230-2239.
231. Jimenez, S.A. and Artlett, C.M. (2005). Microchimerism and systemic sclerosis. *Curr. Opin. Rheumatol.* 17, 86-90.
232. Jimenez, S.A., Feldman, G., Bashey, R.I., Bienkowski, R., and Rosenbloom, J. (1986). Co-ordinate increase in the expression of type I and type III collagen genes in progressive systemic sclerosis fibroblasts. *Biochem. J.* 237, 837-843.
233. Jimenez, S.A., Gaidarova, S., Saitta, B., Sandorfi, N., Herrich, D.J., Rosenbloom, J.C., Kucich, U., Abrams, W.R., and Rosenbloom, J. (2001). Role of protein kinase C-delta in the regulation of collagen gene expression in scleroderma fibroblasts. *J. Clin. Invest* 108, 1395-1403.
234. Jinnin, M., Ihn, H., Asano, Y., Yamane, K., Trojanowska, M., and Tamaki, K. (2004). Tenascin-C upregulation by transforming growth factor-beta in human dermal fibroblasts involves Smad3, Sp1, and Ets1. *Oncogene* 23, 1656-1667.
235. Jinnin, M., Ihn, H., Mimura, Y., Asano, Y., and Tamaki, K. (2006). Potential regulatory elements of the constitutive up-regulated alpha2(I) collagen gene in scleroderma dermal fibroblasts. *Biochem. Biophys. Res. Commun.* 343, 904-909.
236. Jinnin, M., Ihn, H., Yamane, K., Mimura, Y., Asano, Y., and Tamaki, K. (2005). Alpha2(I) collagen gene regulation by protein kinase C signaling in human dermal fibroblasts. *Nucleic Acids Res.* 33, 1337-1351.
237. Johnson, R.W., Tew, M.B., and Arnett, F.C. (2002). The genetics of systemic sclerosis. *Curr. Rheumatol. Rep.* 4, 99-107.

238. Joliot,V., Martinerie,C., Dambrine,G., Plassiart,G., Brisac,M., Crochet,J., and Perbal,B. (1992). Proviral rearrangements and overexpression of a new cellular gene (nov) in myeloblastosis-associated virus type 1-induced nephroblastomas. *Mol. Cell Biol.* 12, 10-21.
239. Kaartinen,V., Voncken,J.W., Shuler,C., Warburton,D., Bu,D., Heisterkamp,N., and Groffen,J. (1995). Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. *Nat. Genet.* 11, 415-421.
240. Kaczynski,J., Cook,T., and Urrutia,R. (2003). Sp1- and Kruppel-like transcription factors. *Genome Biol.* 4, 206.
241. Kadonaga,J.T., Carner,K.R., Masiarz,F.R., and Tjian,R. (1987). Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. *Cell* 51, 1079-1090.
242. Kahaleh,M.B., Sultany,G.L., Smith,E.A., Huffstutter,J.E., Loadholt,C.B., and LeRoy,E.C. (1986). A modified scleroderma skin scoring method. *Clin. Exp. Rheumatol.* 4, 367-369.
243. Kahari,V.M., Chen,Y.Q., Bashir,M.M., Rosenbloom,J., and Uitto,J. (1992). Tumor necrosis factor-alpha down-regulates human elastin gene expression. Evidence for the role of AP-1 in the suppression of promoter activity. *J. Biol. Chem.* 267, 26134-26141.
244. Kahari,V.M., Sandberg,M., Kalimo,H., Vuorio,T., and Vuorio,E. (1988). Identification of fibroblasts responsible for increased collagen production in localized scleroderma by in situ hybridization. *J. Invest Dermatol.* 90, 664-670.
245. Kasai,H., Allen,J.T., Mason,R.M., Kamimura,T., and Zhang,Z. (2005). TGF-beta1 induces human alveolar epithelial to mesenchymal cell transition (EMT). *Respir. Res.* 6, 56.
246. Kawaguchi,Y., Hara,M., and Wright,T.M. (1999). Endogenous IL-1alpha from systemic sclerosis fibroblasts induces IL-6 and PDGF-A. *J. Clin. Invest* 103, 1253-1260.
247. Kawakami,T., Ihn,H., Xu,W., Smith,E., LeRoy,C., and Trojanowska,M. (1998). Increased expression of TGF-beta receptors by scleroderma fibroblasts: evidence for contribution of autocrine TGF-beta signaling to scleroderma phenotype. *J. Invest Dermatol.* 110, 47-51.
248. Kemler,I., Schreiber,E., Muller,M.M., Matthias,P., and Schaffner,W. (1989). Octamer transcription factors bind to two different sequence motifs of the immunoglobulin heavy chain promoter. *EMBO J.* 8, 2001-2008.
249. Kennett,S.B., Udvadia,A.J., and Horowitz,J.M. (1997). Sp3 encodes multiple proteins that differ in their capacity to stimulate or repress transcription. *Nucleic Acids Res.* 25, 3110-3117.
250. Kessler-Becker,D., Smola,S., Krieg,T., and Eckes,B. (2004). High plasminogen activator inhibitor type 2 expression is a hallmark of scleroderma fibroblasts in vitro. *Exp. Dermatol.* 13, 708-714.

251. Kiang,D.T. (1978). Effect of mithramycin on bone beta-glucuronidase and resorption. *Calcif. Tissue Res.* 26, 209-213.
252. Kielty,C.M., Raghunath,M., Siracusa,L.D., Sherratt,M.J., Peters,R., Shuttleworth,C.A., and Jimenez,S.A. (1998). The Tight skin mouse: demonstration of mutant fibrillin-1 production and assembly into abnormal microfibrils. *J. Cell Biol.* 140, 1159-1166.
253. Kikuchi,K., Hartl,C.W., Smith,E.A., LeRoy,E.C., and Trojanowska,M. (1992). Direct demonstration of transcriptional activation of collagen gene expression in systemic sclerosis fibroblasts: insensitivity to TGF beta 1 stimulation. *Biochem. Biophys. Res. Commun.* 187, 45-50.
254. Kikuchi,K., Kadono,T., Ihn,H., Sato,S., Igarashi,A., Nakagawa,H., Tamaki,K., and Takehara,K. (1995). Growth regulation in scleroderma fibroblasts: increased response to transforming growth factor-beta 1. *J. Invest Dermatol.* 105, 128-132.
255. Kim,H.S., Nagalla,S.R., Oh,Y., Wilson,E., Roberts,C.T., Jr., and Rosenfeld,R.G. (1997). Identification of a family of low-affinity insulin-like growth factor binding proteins (IGFBPs): characterization of connective tissue growth factor as a member of the IGFBP superfamily. *Proc. Natl. Acad. Sci. U. S. A* 94, 12981-12986.
256. Kinbara,T., Shirasaki,F., Kawara,S., Inagaki,Y., de Crombrughe,B., and Takehara,K. (2002). Transforming growth factor-beta isoforms differently stimulate proalpha2 (I) collagen gene expression during wound healing process in transgenic mice. *J. Cell Physiol* 190, 375-381.
257. Kingsley,C. and Winoto,A. (1992). Cloning of GT box-binding proteins: a novel Sp1 multigene family regulating T-cell receptor gene expression. *Mol. Cell Biol.* 12, 4251-4261.
258. Kingsley,D.M. (1994). The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev.* 8, 133-146.
259. Kireeva,M.L., Latinkic,B.V., Kolesnikova,T.V., Chen,C.C., Yang,G.P., Abler,A.S., and Lau,L.F. (1997). Cyr61 and Fisp12 are both ECM-associated signaling molecules: activities, metabolism, and localization during development. *Exp. Cell Res.* 233, 63-77.
260. Kirk,T.Z., Mark,M.E., Chua,C.C., Chua,B.H., and Mayes,M.D. (1995). Myofibroblasts from scleroderma skin synthesize elevated levels of collagen and tissue inhibitor of metalloproteinase (TIMP-1) with two forms of TIMP-1. *J. Biol. Chem.* 270, 3423-3428.
261. Kothapalli,D. and Grotendorst,G.R. (2000). CTGF modulates cell cycle progression in cAMP-arrested NRK fibroblasts. *J. Cell Physiol* 182, 119-126.
262. Kretzschmar,M., Doody,J., Timokhina,I., and Massague,J. (1999). A mechanism of repression of TGFbeta/ Smad signaling by oncogenic Ras. *Genes Dev.* 13, 804-816.

263. Krieg,T. and LeRoy,E.C. (1998). Diseases of the extracellular matrix. *J. Mol. Med.* 76, 224-225.
264. Krieg,T., Perlish,J.S., Fleischmajer,R., and Braun-Falco,O. (1985). Collagen synthesis in scleroderma: selection of fibroblast populations during subcultures. *Arch. Dermatol. Res.* 277, 373-376.
265. Kubo,M., Czuwara-Ladykowska,J., Moussa,O., Markiewicz,M., Smith,E., Silver,R.M., Jablonska,S., Blaszczyk,M., Watson,D.K., and Trojanowska,M. (2003). Persistent down-regulation of Fli1, a suppressor of collagen transcription, in fibrotic scleroderma skin. *Am J Pathol* 163, 571-581.
266. Kubota,S., Kawata,K., Yanagita,T., Doi,H., Kitoh,T., and Takigawa,M. (2004). Abundant retention and release of connective tissue growth factor (CTGF/CCN2) by platelets. *J Biochem. (Tokyo)* 136, 279-282.
267. Kubota,S., Mukudai,Y., Moritani,N.H., Nakao,K., Kawata,K., and Takigawa,M. (2005). Translational repression by the cis-acting element of structure-anchored repression (CAESAR) of human ctgf/ccn2 mRNA. *FEBS Lett.* 579, 3751-3758.
268. Kulkarni,A.B., Huh,C.G., Becker,D., Geiser,A., Lyght,M., Flanders,K.C., Roberts,A.B., Sporn,M.B., Ward,J.M., and Karlsson,S. (1993). Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc. Natl. Acad. Sci. U. S. A* 90, 770-774.
269. Kumar,A.P. and Butler,A.P. (1998). Serum responsive gene expression mediated by Sp1. *Biochem. Biophys. Res. Commun.* 252, 517-523.
270. Kuroda,K. and Shinkai,H. (1997). Decorin and glycosaminoglycan synthesis in skin fibroblasts from patients with systemic sclerosis. *Arch. Dermatol. Res.* 289, 481-485.
271. Lagna,G., Hata,A., Hemmati-Brivanlou,A., and Massague,J. (1996). Partnership between DPC4 and SMAD proteins in TGF-beta signalling pathways. *Nature* 383, 832-836.
272. Lania,L., Majello,B., and De Luca,P. (1997). Transcriptional regulation by the Sp family proteins. *Int. J. Biochem. Cell Biol.* 29, 1313-1323.
273. Lasky,J.A., Ortiz,L.A., Tonthat,B., Hoyle,G.W., Corti,M., Athas,G., Lungarella,G., Brody,A., and Friedman,M. (1998). Connective tissue growth factor mRNA expression is upregulated in bleomycin-induced lung fibrosis. *Am J Physiol* 275, L365-L371.
274. Lau,L.F. and Lam,S.C. (1999). The CCN family of angiogenic regulators: the integrin connection. *Exp. Cell Res.* 248, 44-57.
275. Lau,L.F. and Nathans,D. (1985). Identification of a set of genes expressed during the G0/G1 transition of cultured mouse cells. *EMBO J.* 4, 3145-3151.
276. Leask,A. and Abraham,D.J. (2004). TGF-beta signaling and the fibrotic response. *FASEB J.* 18, 816-827.

277. Leask, A. and Abraham, D.J. (2003). The role of connective tissue growth factor, a multifunctional matricellular protein, in fibroblast biology. *Biochem. Cell Biol.* 81, 355-363.
278. Leask, A., Abraham, D.J., Finlay, D.R., Holmes, A., Pennington, D., Shi-wen, X., Chen, Y., Venstrom, K., Dou, X., Ponticos, M., Black, C., Bernabeu, C., Jackman, J.K., Findell, P.R., and Connolly, M.K. (2002a). Dysregulation of transforming growth factor beta signaling in scleroderma: overexpression of endoglin in cutaneous scleroderma fibroblasts. *Arthritis Rheum.* 46, 1857-1865.
279. Leask, A., Holmes, A., and Abraham, D.J. (2002b). Connective tissue growth factor: a new and important player in the pathogenesis of fibrosis. *Curr. Rheumatol. Rep.* 4, 136-142.
280. Leask, A., Holmes, A., Black, C.M., and Abraham, D.J. (2003). Connective tissue growth factor gene regulation. Requirements for its induction by transforming growth factor-beta 2 in fibroblasts. *J. Biol. Chem.* 278, 13008-13015.
281. Lebrin, F., Goumans, M.J., Jonker, L., Carvalho, R.L., Valdimarsdottir, G., Thorikay, M., Mummery, C., Arthur, H.M., and ten Dijke, P. (2004). Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction. *EMBO J.* 23, 4018-4028.
282. Leivonen, S.K., Hakkinen, L., Liu, D., and Kahari, V.M. (2005). Smad3 and extracellular signal-regulated kinase 1/2 coordinately mediate transforming growth factor-beta-induced expression of connective tissue growth factor in human fibroblasts. *J. Invest Dermatol.* 124, 1162-1169.
283. LeRoy, E.C. (1972). Connective tissue synthesis by scleroderma skin fibroblasts in cell culture. *J. Exp. Med.* 135, 1351-1362.
284. LeRoy, E.C. (1974). Increased collagen synthesis by scleroderma skin fibroblasts in vitro: a possible defect in the regulation or activation of the scleroderma fibroblast. *J. Clin. Invest* 54, 880-889.
285. LeRoy, E.C. (1985). Connective tissue disease characterized by fibrosis. In *Textbook of rheumatology*, H.E.R.S. Kelly W, ed. Saunders, Philadelphia), pp. 1183-1205.
286. LeRoy, E.C., Black, C., Fleischmajer, R., Jablonska, S., Krieg, T., Medsger, T.A., Jr., Rowell, N., and Wollheim, F. (1988). Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. *J. Rheumatol.* 15, 202-205.
287. Leroy, E.C., Black, C., Fleischmajer, R., Jablonska, S., Krieg, T., Medsger, T.A., Jr., Rowell, N., and Wollheim, F. (1988). Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. *J. Rheumatol.* 15, 202-205.
288. Leveen, P., Pekny, M., Gebre-Medhin, S., Swolin, B., Larsson, E., and Betsholtz, C. (1994). Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. *Genes Dev.* 8, 1875-1887.
289. Li, C.L., Martinez, V., He, B., Lombet, A., and Perbal, B. (2002). A role for CCN3 (NOV) in calcium signalling. *Mol. Pathol* 55, 250-261.

290. Li, G., Xie, Q., Shi, Y., Li, D., Zhang, M., Jiang, S., Zhou, H., Lu, H., and Jin, Y. (2006). Inhibition of connective tissue growth factor by siRNA prevents liver fibrosis in rats. *J Gene Med.* 8(7), 889-900.
291. Li, L., He, S., Sun, J.M., and Davie, J.R. (2004). Gene regulation by Sp1 and Sp3. *Biochem. Cell Biol.* 82, 460-471.
292. Libby, J., Martinez, R., and Weber, M.J. (1986). Tyrosine phosphorylation in cells treated with transforming growth factor-beta. *J. Cell Physiol* 129, 159-166.
293. Lindahl, G.E., Chambers, R.C., Papakrivopoulou, J., Dawson, S.J., Jacobsen, M.C., Bishop, J.E., and Laurent, G.J. (2002). Activation of fibroblast procollagen alpha 1(I) transcription by mechanical strain is transforming growth factor-beta-dependent and involves increased binding of CCAAT-binding factor (CBF/NF-Y) at the proximal promoter. *J. Biol. Chem.* 277, 6153-6161.
294. Liu, B., Dou, C.L., Prabhu, L., and Lai, E. (1999). FAST-2 is a mammalian winged-helix protein which mediates transforming growth factor beta signals. *Mol. Cell Biol.* 19, 424-430.
295. Liu, X., Sun, Y., Constantinescu, S.N., Karam, E., Weinberg, R.A., and Lodish, H.F. (1997). Transforming growth factor beta-induced phosphorylation of Smad3 is required for growth inhibition and transcriptional induction in epithelial cells. *Proc. Natl. Acad. Sci. U. S. A* 94, 10669-10674.
296. Liu, Y., Kalen, A., Risto, O., and Wahlstrom, O. (2003). Time- and pH-dependent release of PDGF and TGF-beta from platelets in vitro. *Platelets.* 14, 233-237.
297. Lopez-Casillas, F., Wrana, J.L., and Massague, J. (1993). Betaglycan presents ligand to the TGF beta signaling receptor. *Cell* 73, 1435-1444.
298. Ludwicka, A., Ohba, T., Trojanowska, M., Yamakage, A., Strange, C., Smith, E.A., LeRoy, E.C., Sutherland, S., and Silver, R.M. (1995). Elevated levels of platelet derived growth factor and transforming growth factor-beta 1 in bronchoalveolar lavage fluid from patients with scleroderma. *J Rheumatol.* 22, 1876-1883.
299. Lunardi, C., Bason, C., Corrocher, R., and Puccetti, A. (2005). Induction of endothelial cell damage by hCMV molecular mimicry. *Trends Immunol.* 26, 19-24.
300. Ma, L., Lu, M.F., Schwartz, R.J., and Martin, J.F. (2005). Bmp2 is essential for cardiac cushion epithelial-mesenchymal transition and myocardial patterning. *Development* 132, 5601-5611.
301. MacGregor, A.J., Canavan, R., Knight, C., Denton, C.P., Davar, J., Coghlan, J., and Black, C.M. (2001). Pulmonary hypertension in systemic sclerosis: risk factors for progression and consequences for survival. *Rheumatology (Oxford)* 40, 453-459.
302. Macias-Silva, M., Abdollah, S., Hoodless, P.A., Pirone, R., Attisano, L., and Wrana, J.L. (1996). MADR2 is a substrate of the TGFbeta receptor and its phosphorylation is required for nuclear accumulation and signaling. *Cell* 87, 1215-1224.

303. Majello,B., De Luca,P., and Lania,L. (1997). Sp3 is a bifunctional transcription regulator with modular independent activation and repression domains. *J. Biol. Chem.* 272, 4021-4026.
304. Maricq,H.R., Weinrich,M.C., Keil,J.E., Smith,E.A., Harper,F.E., Nussbaum,A.I., LeRoy,E.C., McGregor,A.R., Diat,F., and Rosal,E.J. (1989). Prevalence of scleroderma spectrum disorders in the general population of South Carolina. *Arthritis Rheum.* 32, 998-1006.
305. Marin,M., Karis,A., Visser,P., Grosveld,F., and Philipsen,S. (1997). Transcription factor Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation. *Cell* 89, 619-628.
306. Martin,P. (1997). Wound healing--aiming for perfect skin regeneration. *Science* 276, 75-81.
307. Massague,J. (1998). TGF-beta signal transduction. *Annu. Rev. Biochem.* 67, 753-791.
308. Massague,J. (1990). The transforming growth factor-beta family. *Annu. Rev. Cell Biol.* 6, 597-641.
309. Massague,J. and Wotton,D. (2000). Transcriptional control by the TGF-beta/Smad signaling system. *EMBO J.* 19, 1745-1754.
310. Mastrangelo,I.A., Courey,A.J., Wall,J.S., Jackson,S.P., and Hough,P.V. (1991). DNA looping and Sp1 multimer links: a mechanism for transcriptional synergism and enhancement. *Proc. Natl. Acad. Sci. U. S. A* 88, 5670-5674.
311. Mather,J.P., Moore,A., and Li,R.H. (1997). Activins, inhibins, and follistatins: further thoughts on a growing family of regulators. *Proc. Soc. Exp. Biol. Med.* 215, 209-222.
312. Matsumoto,N., Laub,F., Aldabe,R., Zhang,W., Ramirez,F., Yoshida,T., and Terada,M. (1998). Cloning the cDNA for a new human zinc finger protein defines a group of closely related Kruppel-like transcription factors. *J. Biol. Chem.* 273, 28229-28237.
313. Mattila,L., Airola,K., Ahonen,M., Hietarinta,M., Black,C., Saarialho-Kere,U., and Kahari,V.M. (1998). Activation of tissue inhibitor of metalloproteinases-3 (TIMP-3) mRNA expression in scleroderma skin fibroblasts. *J Invest Dermatol.* 110, 416-421.
314. Mauch,C., Eckes,B., Hunzelmann,N., Oono,T., Kozłowska,E., and Krieg,T. (1993). Control of fibrosis in systemic scleroderma. *J. Invest Dermatol.* 100, 92S-96S.
315. McGaha,T.L., Kodera,T., Spiera,H., Stan,A.C., Pines,M., and Bona,C.A. (2002). Halofuginone inhibition of COL1A2 promoter activity via a c-Jun-dependent mechanism. *Arthritis Rheum.* 46, 2748-2761.
316. McWhirter,A., Colosetti,P., Rubin,K., Miyazono,K., and Black,C. (1994). Collagen type I is not under autocrine control by transforming growth factor-beta 1 in normal and scleroderma fibroblasts. *Lab Invest* 71, 885-894.

317. Menon,R.P., Menon,M.R., Shi-wen,X., Renzoni,E., Bou-Gharios,G., Black,C.M., and Abraham,D.J. (2006). Hammerhead ribozyme-mediated silencing of the mutant fibrillin-1 of tight skin mouse: Insight into the functional role of mutant fibrillin-1. *Exp. Cell Res.* 312, 1463-1474.
318. Merchant,J.L., Du,M., and Todisco,A. (1999). Sp1 phosphorylation by Erk 2 stimulates DNA binding. *Biochem. Biophys. Res. Commun.* 254, 454-461.
319. Mercurio,S., Latinkic,B., Itasaki,N., Krumlauf,R., and Smith,J.C. (2004). Connective-tissue growth factor modulates WNT signalling and interacts with the WNT receptor complex. *Development* 131, 2137-2147.
320. Micera,A., Vigneti,E., Pickholtz,D., Reich,R., Pappo,O., Bonini,S., Maquart,F.X., Aloe,L., and Levi-Schaffer,F. (2001). Nerve growth factor displays stimulatory effects on human skin and lung fibroblasts, demonstrating a direct role for this factor in tissue repair. *Proc. Natl. Acad. Sci. U. S. A* 98, 6162-6167.
321. Midgley,V.C. and Khachigian,L.M. (2004). Fibroblast growth factor-2 induction of platelet-derived growth factor-C chain transcription in vascular smooth muscle cells is ERK-dependent but not JNK-dependent and mediated by Egr-1. *J. Biol. Chem.* 279, 40289-40295.
322. Miller,D.M., Polansky,D.A., Thomas,S.D., Ray,R., Campbell,V.W., Sanchez,J., and Koller,C.A. (1987). Mithramycin selectively inhibits transcription of G-C containing DNA. *Am. J. Med. Sci.* 294, 388-394.
323. Mimura,Y., Ihn,H., Jinnin,M., Asano,Y., Yamane,K., and Tamaki,K. (2005). Constitutive thrombospondin-1 overexpression contributes to autocrine transforming growth factor-beta signaling in cultured scleroderma fibroblasts. *Am. J. Pathol.* 166, 1451-1463.
324. Mizuide,M., Hara,T., Furuya,T., Takeda,M., Kusanagi,K., Inada,Y., Mori,M., Imamura,T., Miyazawa,K., and Miyazono,K. (2003). Two short segments of Smad3 are important for specific interaction of Smad3 with c-Ski and SnoN. *J Biol. Chem.* 278, 531-536.
325. Mo,F.E., Muntean,A.G., Chen,C.C., Stolz,D.B., Watkins,S.C., and Lau,L.F. (2002). CYR61 (CCN1) is essential for placental development and vascular integrity. *Mol. Cell Biol.* 22, 8709-8720.
326. Moorefield,K.S., Fry,S.J., and Horowitz,J.M. (2004). Sp2 DNA binding activity and trans-activation are negatively regulated in mammalian cells. *J. Biol. Chem.* 279, 13911-13924.
327. Mori,S., Matsuzaki,K., Yoshida,K., Furukawa,F., Tahashi,Y., Yamagata,H., Sekimoto,G., Seki,T., Matsui,H., Nishizawa,M., Fujisawa,J., and Okazaki,K. (2004). TGF-beta and HGF transmit the signals through JNK-dependent Smad2/3 phosphorylation at the linker regions. *Oncogene* 23, 7416-7429.
328. Mori,T., Kawara,S., Shinozaki,M., Hayashi,N., Kakinuma,T., Igarashi,A., Takigawa,M., Nakanishi,T., and Takehara,K. (1999). Role and interaction of connective tissue growth factor with transforming growth factor-beta in persistent fibrosis: A mouse fibrosis model. *J. Cell Physiol* 181, 153-159.

329. Mori,Y., Chen,S.J., and Varga,J. (2003). Expression and regulation of intracellular SMAD signaling in scleroderma skin fibroblasts. *Arthritis Rheum.* 48, 1964-1978.
330. Moritani,N.H., Kubota,S., Nishida,T., Kawaki,H., Kondo,S., Sugahara,T., and Takigawa,M. (2003). Suppressive effect of overexpressed connective tissue growth factor on tumor cell growth in a human oral squamous cell carcinoma-derived cell line. *Cancer Lett.* 192, 205-214.
331. Morrissey,E.E. (2003). Wnt signaling and pulmonary fibrosis. *Am J Pathol* 162, 1393-1397.
332. Motojima,M., Ando,T., and Yoshioka,T. (2000). Sp1-like activity mediates angiotensin-II-induced plasminogen-activator inhibitor type-1 (PAI-1) gene expression in mesangial cells. *Biochem. J.* 349, 435-441.
333. Moussad,E.E. and Brigstock,D.R. (2000). Connective tissue growth factor: what's in a name? *Mol. Genet. Metab* 71, 276-292.
334. Moustakas,A. and Heldin,C.H. (2005). Non-Smad TGF- β signals. *J. Cell Sci.* 118, 3573-3584.
335. Moustakas,A., Pardali,K., Gaal,A., and Heldin,C.H. (2002). Mechanisms of TGF- β signaling in regulation of cell growth and differentiation. *Immunol. Lett.* 82, 85-91.
336. Mu,D., Cambier,S., Fjellbirkeland,L., Baron,J.L., Munger,J.S., Kawakatsu,H., Sheppard,D., Broaddus,V.C., and Nishimura,S.L. (2002). The integrin $\alpha(v)\beta 8$ mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF- $\beta 1$. *J. Cell Biol.* 157, 493-507.
337. Mukerjee,D., St George,D., Coleiro,B., Knight,C., Denton,C.P., Davar,J., Black,C.M., and Coghlan,J.G. (2003). Prevalence and outcome in systemic sclerosis associated pulmonary arterial hypertension: application of a registry approach. *Ann. Rheum. Dis.* 62, 1088-1093.
338. Munz,B., Tretter,Y.P., Hertel,M., Engelhardt,F., Alzheimer,C., and Werner,S. (2001). The roles of activins in repair processes of the skin and the brain. *Mol. Cell Endocrinol.* 180, 169-177.
339. Murphy-Ullrich,J.E. and Poczatek,M. (2000). Activation of latent TGF- β by thrombospondin-1: mechanisms and physiology. *Cytokine Growth Factor Rev.* 11, 59-69.
340. Mutsaers,S.E., Bishop,J.E., McGrouther,G., and Laurent,G.J. (1997). Mechanisms of tissue repair: from wound healing to fibrosis. *Int. J Biochem. Cell Biol.* 29, 5-17.
341. Nagarajan,R.P., Zhang,J., Li,W., and Chen,Y. (1999). Regulation of Smad7 promoter by direct association with Smad3 and Smad4. *J. Biol. Chem.* 274, 33412-33418.
342. Nakanishi,T., Nishida,T., Shimo,T., Kobayashi,K., Kubo,T., Tamatani,T., Tezuka,K., and Takigawa,M. (2000). Effects of CTGF/Hcs24, a product of a

- hypertrophic chondrocyte-specific gene, on the proliferation and differentiation of chondrocytes in culture. *Endocrinology* 141, 264-273.
343. Nakanishi,T., Yamaai,T., Asano,M., Nawachi,K., Suzuki,M., Sugimoto,T., and Takigawa,M. (2001). Overexpression of connective tissue growth factor/hypertrophic chondrocyte-specific gene product 24 decreases bone density in adult mice and induces dwarfism. *Biochem. Biophys. Res. Commun.* 281, 678-681.
 344. Nakao,A., Afrakhte,M., Moren,A., Nakayama,T., Christian,J.L., Heuchel,R., Itoh,S., Kawabata,M., Heldin,N.E., Heldin,C.H., and ten Dijke,P. (1997). Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. *Nature* 389, 631-635.
 345. Nakao,A., Fujii,M., Matsumura,R., Kumano,K., Saito,Y., Miyazono,K., and Iwamoto,I. (1999). Transient gene transfer and expression of Smad7 prevents bleomycin-induced lung fibrosis in mice. *J. Clin. Invest* 104, 5-11.
 346. Nakerakanti,S.S., Kapanadze,B., Yamasaki,M., Markiewicz,M., and Trojanowska,M. (2006). FLI1 and Ets1 have distinct roles in the CTGF/CCN2 gene regulation and induction of the profibrotic gene program. *J Biol. Chem.* 281(35), 25259-25269.
 347. Needleman,B.W., Choi,J., Burrows-Mezu,A., and Fontana,J.A. (1990). Secretion and binding of transforming growth factor beta by scleroderma and normal dermal fibroblasts. *Arthritis Rheum.* 33, 650-656.
 348. Nehls,M.C., Grapilon,M.L., and Brenner,D.A. (1992). NF-I/Sp1 switch elements regulate collagen alpha 1(I) gene expression. *DNA Cell Biol.* 11, 443-452.
 349. Nehls,M.C., Rippe,R.A., Veloz,L., and Brenner,D.A. (1991). Transcription factors nuclear factor I and Sp1 interact with the murine collagen alpha 1 (I) promoter. *Mol. Cell Biol.* 11, 4065-4073.
 350. Nelson,J.L., Furst,D.E., Maloney,S., Gooley,T., Evans,P.C., Smith,A., Bean,M.A., Ober,C., and Bianchi,D.W. (1998). Microchimerism and HLA-compatible relationships of pregnancy in scleroderma. *Lancet* 351, 559-562.
 351. Nguyen,V.A., Sgonc,R., Dietrich,H., and Wick,G. (2000). Endothelial injury in internal organs of University of California at Davis line 200 (UCD 200) chickens, an animal model for systemic sclerosis (Scleroderma). *J. Autoimmun.* 14, 143-149.
 352. Nietert,P.J. and Silver,R.M. (2000). Systemic sclerosis: environmental and occupational risk factors. *Curr. Opin. Rheumatol.* 12, 520-526.
 353. Nietert,P.J., Silver,R.M., Mitchell,H.C., Shaftman,S.R., and Tilley,B.C. (2005). Demographic and clinical factors associated with in-hospital death among patients with systemic sclerosis. *J Rheumatol.* 32, 1888-1892.
 354. Nishida,T., Kubota,S., Fukunaga,T., Kondo,S., Yosimichi,G., Nakanishi,T., Takano-Yamamoto,T., and Takigawa,M. (2003). CTGF/Hcs24, hypertrophic chondrocyte-specific gene product, interacts with perlecan in regulating the proliferation and differentiation of chondrocytes. *J. Cell Physiol* 196, 265-275.

355. Nishihara,A., Hanai,J.I., Okamoto,N., Yanagisawa,J., Kato,S., Miyazono,K., and Kawabata,M. (1998). Role of p300, a transcriptional coactivator, in signalling of TGF-beta. *Genes Cells* 3, 613-623.
356. Nishimoto,S. and Nishida,E. (2006). MAPK signalling: ERK5 versus ERK1/2. *EMBO Rep.* 7, 782-786.
357. Nohe,A., Hassel,S., Ehrlich,M., Neubauer,F., Sebald,W., Henis,Y.I., and Knaus,P. (2002). The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways. *J. Biol. Chem.* 277, 5330-5338.
358. Oemar,B.S., Werner,A., Garnier,J.M., Do,D.D., Godoy,N., Nauck,M., Marz,W., Rupp,J., Pech,M., and Luscher,T.F. (1997). Human connective tissue growth factor is expressed in advanced atherosclerotic lesions. *Circulation* 95, 831-839.
359. Omoteyama,K., Ikeda,H., Imaki,J., and Sakai,M. (2006). Activation of connective tissue growth factor gene by the c-Maf and Lc-Maf transcription factors. *Biochem. Biophys. Res. Commun.* 339, 1089-1097.
360. Pan,L.H., Beppu,T., Kurose,A., Yamauchi,K., Sugawara,A., Suzuki,M., Ogawa,A., and Sawai,T. (2002). Neoplastic cells and proliferating endothelial cells express connective tissue growth factor (CTGF) in glioblastoma. *Neurol. Res.* 24, 677-683.
361. Pan,L.H., Yamauchi,K., Uzuki,M., Nakanishi,T., Takigawa,M., Inoue,H., and Sawai,T. (2001). Type II alveolar epithelial cells and interstitial fibroblasts express connective tissue growth factor in IPF. *Eur. Respir. J* 17, 1220-1227.
362. Pannu,J., Gore-Hyer,E., Yamanaka,M., Smith,E.A., Rubinchik,S., Dong,J.Y., Jablonska,S., Blaszczyk,M., and Trojanowska,M. (2004). An increased transforming growth factor beta receptor type I:type II ratio contributes to elevated collagen protein synthesis that is resistant to inhibition via a kinase-deficient transforming growth factor beta receptor type II in scleroderma. *Arthritis Rheum.* 50, 1566-1577.
363. Paradis,V., Dargere,D., Vidaud,M., de Gouville,A.C., Huet,S., Martinez,V., Gauthier,J.M., Ba,N., Sobesky,R., Ratzu,V., and Bedossa,P. (1999). Expression of connective tissue growth factor in experimental rat and human liver fibrosis. *Hepatology* 30, 968-976.
364. Pardali,K., Kurisaki,A., Moren,A., ten Dijke,P., Kardassis,D., and Moustakas,A. (2000). Role of Smad proteins and transcription factor Sp1 in p21(Waf1/Cip1) regulation by transforming growth factor-beta. *J. Biol. Chem.* 275, 29244-29256.
365. Park,S.K., Kim,J., Seomun,Y., Choi,J., Kim,D.H., Han,I.O., Lee,E.H., Chung,S.K., and Joo,C.K. (2001). Hydrogen peroxide is a novel inducer of connective tissue growth factor. *Biochem. Biophys. Res. Commun.* 284, 966-971.
366. Patel,R. and Kos,L. (2005). Endothelin-1 and Neuregulin-1 convert embryonic cardiomyocytes into cells of the conduction system in the mouse. *Dev. Dyn.* 233, 20-28.

367. Perbal,B. (2004). CCN proteins: multifunctional signalling regulators. *Lancet* 363, 62-64.
368. Perbal,B. (1999). Nuclear localisation of NOVH protein: a potential role for NOV in the regulation of gene expression. *Mol. Pathol.* 52, 84-91.
369. Perbal,B., Brigstock,D.R., and Lau,L.F. (2003). Report on the second international workshop on the CCN family of genes. *Mol. Pathol.* 56, 80-85.
370. Persson,U., Izumi,H., Souchelnytskyi,S., Itoh,S., Grimsby,S., Engstrom,U., Heldin,C.H., Funahara,K., and ten Dijke,P. (1998). The L45 loop in type I receptors for TGF-beta family members is a critical determinant in specifying Smad isoform activation. *FEBS Lett.* 434, 83-87.
371. Pessah,M., Prunier,C., Marais,J., Ferrand,N., Mazars,A., Lallemand,F., Gauthier,J.M., and Atfi,A. (2001). c-Jun interacts with the corepressor TG-interacting factor (TGIF) to suppress Smad2 transcriptional activity. *Proc. Natl. Acad. Sci. U. S. A* 98, 6198-6203.
372. Phanish,M.K., Wahab,N.A., Hendry,B.M., and Dockrell,M.E. (2005). TGF-beta1-induced connective tissue growth factor (CCN2) expression in human renal proximal tubule epithelial cells requires Ras/MEK/ERK and Smad signalling. *Nephron Exp. Nephrol.* 100, e156-e165.
373. Phelps,R.G., Daian,C., Shibata,S., Fleischmajer,R., and Bona,C.A. (1993). Induction of skin fibrosis and autoantibodies by infusion of immunocompetent cells from tight skin mice into C57BL/6 Pa/Pa mice. *J. Autoimmun.* 6, 701-718.
374. Pizzo,A.M., Kokini,K., Vaughn,L.C., Waisner,B.Z., and Voytik-Harbin,S.L. (2005). Extracellular matrix (ECM) microstructural composition regulates local cell-ECM biomechanics and fundamental fibroblast behavior: a multidimensional perspective. *J Appl. Physiol* 98, 1909-1921.
375. Pogulis,R.J. and Freytag,S.O. (1993). Contribution of specific cis-acting elements to activity of the mouse pro-alpha 2(I) collagen enhancer. *J. Biol. Chem.* 268, 2493-2499.
376. Poncelet,A.C. and Schnaper,H.W. (2001). Sp1 and Smad proteins cooperate to mediate transforming growth factor-beta 1-induced alpha 2(I) collagen expression in human glomerular mesangial cells. *J. Biol. Chem.* 276, 6983-6992.
377. Ponticos,M., Abraham,D., Alexakis,C., Lu,Q.L., Black,C., Partridge,T., and Bou-Gharios,G. (2004). Col1a2 enhancer regulates collagen activity during development and in adult tissue repair. *Matrix Biol.* 22, 619-628.
378. Pontrelli,P., Ranieri,E., Ursi,M., Ghosh-Choudhury,G., Gesualdo,L., Paolo,S.F., and Grandaliano,G. (2004). jun-N-terminal kinase regulates thrombin-induced PAI-1 gene expression in proximal tubular epithelial cells. *Kidney Int.* 65, 2249-2261.
379. Postlethwaite,A.E., Keski-Oja,J., Moses,H.L., and Kang,A.H. (1987). Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor beta. *J. Exp. Med.* 165, 251-256.

380. Prescott,R.J., Freemont,A.J., Jones,C.J., Hoyland,J., and Fielding,P. (1992). Sequential dermal microvascular and perivascular changes in the development of scleroderma. *J Pathol* 166, 255-263.
381. Prockop,D.J. and Kivirikko,K.I. (1995). Collagens: molecular biology, diseases, and potentials for therapy. *Annu. Rev. Biochem.* 64, 403-434.
382. Proetzel,G., Pawlowski,S.A., Wiles,M.V., Yin,M., Boivin,G.P., Howles,P.N., Ding,J., Ferguson,M.W., and Doetschman,T. (1995). Transforming growth factor-beta 3 is required for secondary palate fusion. *Nat. Genet.* 11, 409-414.
383. Qian,S.W., Burmester,J.K., Tsang,M.L., Weatherbee,J.A., Hinck,A.P., Ohlsen,D.J., Sporn,M.B., and Roberts,A.B. (1996). Binding affinity of transforming growth factor-beta for its type II receptor is determined by the C-terminal region of the molecule. *J. Biol. Chem.* 271, 30656-30662.
384. Querfeld,C., Eckes,B., Huerkamp,C., Krieg,T., and Sollberg,S. (1999). Expression of TGF-beta 1, -beta 2 and -beta 3 in localized and systemic scleroderma. *J. Dermatol. Sci.* 21, 13-22.
385. Rajkumar,V.S., Howell,K., Csiszar,K., Denton,C.P., Black,C.M., and Abraham,D.J. (2005). Shared expression of phenotypic markers in systemic sclerosis indicates a convergence of pericytes and fibroblasts to a myofibroblast lineage in fibrosis. *Arthritis Res. Ther.* 7, R1113-R1123.
386. Rajkumar,V.S., Sundberg,C., Abraham,D.J., Rubin,K., and Black,C.M. (1999). Activation of microvascular pericytes in autoimmune Raynaud's phenomenon and systemic sclerosis. *Arthritis Rheum.* 42, 930-941.
387. Remberger,M., Jaksch,M., Uzunel,M., and Mattsson,J. (2003). Serum levels of cytokines correlate to donor chimerism and acute graft-vs.-host disease after haematopoietic stem cell transplantation. *Eur. J. Haematol.* 70, 384-391.
388. Reveille,J.D., Fischbach,M., McNearney,T., Friedman,A.W., Aguilar,M.B., Lisse,J., Fritzler,M.J., Ahn,C., and Arnett,F.C. (2001). Systemic sclerosis in 3 US ethnic groups: a comparison of clinical, sociodemographic, serologic, and immunogenetic determinants. *Semin. Arthritis Rheum.* 30, 332-346.
389. Rifkin,D.B. (2005). Latent transforming growth factor-beta (TGF-beta) binding proteins: orchestrators of TGF-beta availability. *J. Biol. Chem.* 280, 7409-7412.
390. Rippe,R.A., Almounajed,G., and Brenner,D.A. (1995). Sp1 binding activity increases in activated Ito cells. *Hepatology* 22, 241-251.
391. Riser,B.L., Cortes,P., deNichilo,M., Deshmukh,P.V., Chahal,P.S., Mohammed,A.K., Yee,J., and Kahkonen,D. (2003). Urinary CCN2 (CTGF) as a possible predictor of diabetic nephropathy: preliminary report. *Kidney Int.* 64, 451-458.
392. Riser,B.L., deNichilo,M., Cortes,P., Baker,C., Grondin,J.M., Yee,J., and Narins,R.G. (2000). Regulation of connective tissue growth factor activity in cultured rat mesangial cells and its expression in experimental diabetic glomerulosclerosis. *J Am Soc. Nephrol.* 11, 25-38.

393. Roberts,A.B., Anzano,M.A., Lamb,L.C., Smith,J.M., and Sporn,M.B. (1981). New class of transforming growth factors potentiated by epidermal growth factor: isolation from non-neoplastic tissues. *Proc. Natl. Acad. Sci. U. S. A* 78, 5339-5343.
394. Roberts,A.B., Kim,S.J., Noma,T., Glick,A.B., Lafyatis,R., Lechleider,R., Jakowlew,S.B., Geiser,A., O'Reilly,M.A., and Danielpour,D. (1991). Multiple forms of TGF-beta: distinct promoters and differential expression. *Ciba Found. Symp.* 157, 7-15.
395. Roestenberg,P., van Nieuwenhoven,F.A., Joles,J.A., Trischberger,C., Martens,P.P., Oliver,N., Aten,J., Hoppener,J.W., and Goldschmeding,R. (2005). Temporal expression profile and distribution pattern indicate a role of connective tissue growth factor (CTGF/CCN-2) in diabetic nephropathy in mice. *Am. J. Physiol Renal Physiol.*
396. Rossouw,C.M., Vergeer,W.P., du Plooy,S.J., Bernard,M.P., Ramirez,F., and de Wet,W.J. (1987). DNA sequences in the first intron of the human pro-alpha 1(I) collagen gene enhance transcription. *J. Biol. Chem.* 262, 15151-15157.
397. Rudnicka,L., Varga,J., Christiano,A.M., Iozzo,R.V., Jimenez,S.A., and Uitto,J. (1994). Elevated expression of type VII collagen in the skin of patients with systemic sclerosis. Regulation by transforming growth factor-beta. *J. Clin. Invest* 93, 1709-1715.
398. Ryseck,R.P., Macdonald-Bravo,H., Mattei,M.G., and Bravo,R. (1991). Structure, mapping, and expression of fisp-12, a growth factor-inducible gene encoding a secreted cysteine-rich protein. *Cell Growth Differ.* 2, 225-233.
399. Ryu,H., Lee,J., Olofsson,B.A., Mwidau,A., Dedeoglu,A., Escudero,M., Flemington,E., Azizkhan-Clifford,J., Ferrante,R.J., and Ratan,R.R. (2003). Histone deacetylase inhibitors prevent oxidative neuronal death independent of expanded polyglutamine repeats via an Sp1-dependent pathway. *Proc. Natl. Acad. Sci. U. S. A* 100, 4281-4286.
400. Saito,S., Kasturi,K., and Bona,C. (1999a). Genetic and immunologic features associated with scleroderma-like syndrome of TSK mice. *Curr. Rheumatol. Rep.* 1, 34-37.
401. Saito,S., Nishimura,H., Brumeanu,T.D., Casares,S., Stan,A.C., Honjo,T., and Bona,C.A. (1999b). Characterization of mutated protein encoded by partially duplicated fibrillin-1 gene in tight skin (TSK) mice. *Mol. Immunol.* 36, 169-176.
402. Saito,S., Nishimura,H., Phelps,R.G., Wolf,I., Suzuki,M., Honjo,T., and Bona,C. (2000). Induction of skin fibrosis in mice expressing a mutated fibrillin-1 gene. *Mol. Med.* 6, 825-836.
403. Saitta,B., Gaidarova,S., Cicchillitti,L., and Jimenez,S.A. (2000). CCAAT binding transcription factor binds and regulates human COL1A1 promoter activity in human dermal fibroblasts: demonstration of increased binding in systemic sclerosis fibroblasts. *Arthritis Rheum.* 43, 2219-2229.
404. Sanchez-Elsner,T., Ramirez,J.R., Sanz-Rodriguez,F., Varela,E., Bernabeu,C., and Botella,L.M. (2004). A cross-talk between hypoxia and TGF-beta

orchestrates erythropoietin gene regulation through SP1 and Smads. *J. Mol. Biol.* 336, 9-24.

405. Sanford, L.P., Ormsby, I., Gittenberger-de Groot, A.C., Sariola, H., Friedman, R., Boivin, G.P., Cardell, E.L., and Doetschman, T. (1997). TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development* 124, 2659-2670.
406. Santander, C. and Brandan, E. (2006). Betaglycan induces TGF-beta signaling in a ligand-independent manner, through activation of the p38 pathway. *Cell Signal.*
407. Sato, M., Shegogue, D., Gore, E.A., Smith, E.A., McDermott, P.J., and Trojanowska, M. (2002). Role of p38 MAPK in transforming growth factor beta stimulation of collagen production by scleroderma and healthy dermal fibroblasts. *J. Invest Dermatol.* 118, 704-711.
408. Sato, S., Hayakawa, I., Hasegawa, M., Fujimoto, M., and Takehara, K. (2003). Function blocking autoantibodies against matrix metalloproteinase-1 in patients with systemic sclerosis. *J Invest Dermatol.* 120, 542-547.
409. Sato, S., Nagaoka, T., Hasegawa, M., Tamatani, T., Nakanishi, T., Takigawa, M., and Takehara, K. (2000). Serum levels of connective tissue growth factor are elevated in patients with systemic sclerosis: association with extent of skin sclerosis and severity of pulmonary fibrosis. *J. Rheumatol.* 27, 149-154.
410. Scarpati, E.M. and DiCorleto, P.E. (1996). Identification of a thrombin response element in the human platelet-derived growth factor B-chain (c-sis) promoter. *J. Biol. Chem.* 271, 3025-3032.
411. Schild, C. and Trueb, B. (2002). Mechanical stress is required for high-level expression of connective tissue growth factor. *Exp. Cell Res.* 274, 83-91.
412. Schiller, M., Javelaud, D., and Mauviel, A. (2004). TGF-beta-induced SMAD signaling and gene regulation: consequences for extracellular matrix remodeling and wound healing. *J. Dermatol. Sci.* 35, 83-92.
413. Schmierer, B. and Hill, C.S. (2005). Kinetic analysis of Smad nucleocytoplasmic shuttling reveals a mechanism for transforming growth factor beta-dependent nuclear accumulation of Smads. *Mol. Cell Biol.* 25, 9845-9858.
414. Schober, J.M., Chen, N., Grzeszkiewicz, T.M., Jovanovic, I., Emeson, E.E., Ugarova, T.P., Ye, R.D., Lau, L.F., and Lam, S.C. (2002). Identification of integrin alpha(M)beta(2) as an adhesion receptor on peripheral blood monocytes for Cyr61 (CCN1) and connective tissue growth factor (CCN2): immediate-early gene products expressed in atherosclerotic lesions. *Blood* 99, 4457-4465.
415. Schwab, J.M., Postler, E., Nguyen, T.D., Mittelbronn, M., Meyermann, R., and Schluesener, H.J. (2000). Connective tissue growth factor is expressed by a subset of reactive astrocytes in human cerebral infarction. *Neuropathol. Appl. Neurobiol.* 26, 434-440.
416. Segarini, P.R., Nesbitt, J.E., Li, D., Hays, L.G., Yates, J.R., III, and Carmichael, D.F. (2001). The low density lipoprotein receptor-related

protein/alpha2-macroglobulin receptor is a receptor for connective tissue growth factor. *J Biol. Chem.* 276, 40659-40667.

417. Seth,A. and Watson,D.K. (2005). ETS transcription factors and their emerging roles in human cancer. *Eur. J Cancer* 41, 2462-2478.
418. Sfrikakis,P.P., McCune,B.K., Tsokos,M., Aroni,K., Vayiopoulos,G., and Tsokos,G.C. (1993). Immunohistological demonstration of transforming growth factor-beta isoforms in the skin of patients with systemic sclerosis. *Clin. Immunol. Immunopathol.* 69, 199-204.
419. Sgonc,R., Gruschwitz,M.S., Boeck,G., Sepp,N., Gruber,J., and Wick,G. (2000). Endothelial cell apoptosis in systemic sclerosis is induced by antibody-dependent cell-mediated cytotoxicity via CD95. *Arthritis Rheum.* 43, 2550-2562.
420. Shakunaga,T., Ozaki,T., Ohara,N., Asaumi,K., Doi,T., Nishida,K., Kawai,A., Nakanishi,T., Takigawa,M., and Inoue,H. (2000). Expression of connective tissue growth factor in cartilaginous tumors. *Cancer* 89, 1466-1473.
421. Shepro,D. and Morel,N.M. (1993). Pericyte physiology. *FASEB J.* 7, 1031-1038.
422. Shi, Y., Wang,Y.F., Jayaraman,L., Yang,H., Massague,J., and Pavletich,N.P. (1998). Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-beta signaling. *Cell* 94, 585-594.
423. Shi-wen,X., Chen,Y., Denton,C.P., Eastwood,M., Renzoni,E.A., Bou-Gharios,G., Pearson,J.D., Dashwood,M., du Bois,R.M., Black,C.M., Leask,A., and Abraham,D.J. (2004). Endothelin-1 promotes myofibroblast induction through the ETA receptor via a rac/phosphoinositide 3-kinase/Akt-dependent pathway and is essential for the enhanced contractile phenotype of fibrotic fibroblasts. *Mol. Biol. Cell* 15, 2707-2719.
424. Shi-wen,X., Pennington,D., Holmes,A., Leask,A., Bradham,D., Beauchamp,J.R., Fonseca,C., du Bois,R.M., Martin,G.R., Black,C.M., and Abraham,D.J. (2000). Autocrine overexpression of CTGF maintains fibrosis: RDA analysis of fibrosis genes in systemic sclerosis. *Exp. Cell Res.* 259, 213-224.
425. Shi-wen,X., Rodriguez-Pascual,F., Lamas,S., Holmes,A., Howat,S., Pearson,J.D., Dashwood,M.R., du Bois,R.M., Denton,C.P., Black,C.M., Abraham,D.J., and Leask,A. (2006a). Constitutive ALK5-Independent c-Jun N-Terminal Kinase Activation Contributes to Endothelin-1 Overexpression in Pulmonary Fibrosis: Evidence of an Autocrine Endothelin Loop Operating through the Endothelin A and B Receptors. *Mol. Cell Biol.* 26, 5518-5527.
426. Shi-wen,X., Stanton,L.A., Kennedy,L., Pala,D., Chen,Y., Howat,S.L., Renzoni,E.A., Carter,D.E., Bou-Gharios,G., Stratton,R.J., Pearson,J.D., Beier,F., Lyons,K.M., Black,C.M., Abraham,D.J., and Leask,A. (2006b). CCN2 Is Necessary for Adhesive Responses to Transforming Growth Factor-beta1 in Embryonic Fibroblasts. *J. Biol. Chem.* 281, 10715-10726.

427. Shimo,T., Koyama,E., Sugito,H., Wu,C., Shimo,S., and Pacifici,M. (2005). Retinoid signaling regulates CTGF expression in hypertrophic chondrocytes with differential involvement of MAP kinases. *J. Bone Miner. Res.* 20, 867-877.
428. Shimo,T., Nakanishi,T., Nishida,T., Asano,M., Kanyama,M., Kuboki,T., Tamatani,T., Tezuka,K., Takemura,M., Matsumura,T., and Takigawa,M. (1999). Connective tissue growth factor induces the proliferation, migration, and tube formation of vascular endothelial cells in vitro, and angiogenesis in vivo. *J Biochem. (Tokyo)* 126, 137-145.
429. Silman,A.J. and Newman,J. (1996). Epidemiology of systemic sclerosis. *Curr. Opin. Rheumatol.* 8, 585-589.
430. Silver,R. (1996a). Scleroderma and Pseudoscleroderma: Environmental Exposure. In *Systemic Sclerosis*, P.J.Clements and D.E.Furst, eds. Williams & Wilkins, Baltimore), pp. 81-98.
431. Silver,R.M. (1996b). Scleroderma and Pseudoscleroderma: Environmental Exposure. In *Systemic Sclerosis*, Clements P.J and Furst D.E, eds. Williams & Wilkins, Baltimore), pp. 81-98.
432. Singer,A.J. and Clark,R.A. (1999). Cutaneous wound healing. *N. Engl. J. Med.* 341, 738-746.
433. Smith,E.A. and LeRoy,E.C. (1990). A possible role for transforming growth factor-beta in systemic sclerosis. *J. Invest Dermatol.* 95, 125S-127S.
434. Snowden,N., Coupes,B., Herrick,A., Illingworth,K., Jayson,M.I., and Brenchley,P.E. (1994). Plasma TGF beta in systemic sclerosis: a cross-sectional study. *Ann. Rheum. Dis.* 53, 763-767.
435. Sonnylal S and de Crombrughe, B. Mice expression a conditional fibroblast specific constitutively active TGF-beta receptor type I recapitulates the histological and biochemical features of systemic sclerosis. Meeting abstract- Pathogenic mechanisms of fibrosis:search for common ground . 1-10-2004. Ref Type: Abstract
436. Sporn,M.B. and Roberts,A.B. (1992). Transforming growth factor-beta: recent progress and new challenges. *J. Cell Biol.* 119, 1017-1021.
437. Sporn,M.B., Roberts,A.B., Wakefield,L.M., and Assoian,R.K. (1986). Transforming growth factor-beta: biological function and chemical structure. *Science* 233, 532-534.
438. Stanhope-Baker,P. and Williams,B.R. (2000). Identification of connective tissue growth factor as a target of WT1 transcriptional regulation. *J. Biol. Chem.* 275, 38139-38150.
439. Steen,V.D., Conte,C., Owens,G.R., and Medsger,T.A., Jr. (1994). Severe restrictive lung disease in systemic sclerosis. *Arthritis Rheum.* 37, 1283-1289.
440. Steen,V.D. and Medsger,T.A., Jr. (2000). Severe organ involvement in systemic sclerosis with diffuse scleroderma. *Arthritis Rheum.* 43, 2437-2444.

441. Stenvers,K.L., Tursky,M.L., Harder,K.W., Kountouri,N., Amatayakul-Chantler,S., Grail,D., Small,C., Weinberg,R.A., Sizeland,A.M., and Zhu,H.J. (2003). Heart and liver defects and reduced transforming growth factor beta2 sensitivity in transforming growth factor beta type III receptor-deficient embryos. *Mol. Cell Biol.* 23, 4371-4385.
442. Stratton,R., Rajkumar,V., Ponticos,M., Nichols,B., Shiwen,X., Black,C.M., Abraham,D.J., and Leask,A. (2002). Prostacyclin derivatives prevent the fibrotic response to TGF-beta by inhibiting the Ras/MEK/ERK pathway. *FASEB J.* 16, 1949-1951.
443. Stratton,R., Shiwen,X., Martini,G., Holmes,A., Leask,A., Haberberger,T., Martin,G.R., Black,C.M., and Abraham,D. (2001). Iloprost suppresses connective tissue growth factor production in fibroblasts and in the skin of scleroderma patients. *J. Clin. Invest* 108, 241-250.
444. Suter,L.G., Murabito,J.M., Felson,D.T., and Fraenkel,L. (2005). The incidence and natural history of Raynaud's phenomenon in the community. *Arthritis Rheum.* 52, 1259-1263.
445. Suzuki,T., Kimura,A., Nagai,R., and Horikoshi,M. (2000). Regulation of interaction of the acetyltransferase region of p300 and the DNA-binding domain of Sp1 on and through DNA binding. *Genes Cells* 5, 29-41.
446. Suzuma,K., Naruse,K., Suzuma,I., Takahara,N., Ueki,K., Aiello,L.P., and King,G.L. (2000). Vascular endothelial growth factor induces expression of connective tissue growth factor via KDR, Flt1, and phosphatidylinositol 3-kinase-akt-dependent pathways in retinal vascular cells. *J. Biol. Chem.* 275, 40725-40731.
447. Svegliati,S., Canello,R., Sambo,P., Luchetti,M., Paroncini,P., Orlandini,G., Discepoli,G., Paterno,R., Santillo,M., Cuozzo,C., Cassano,S., Avvedimento,E.V., and Gabrielli,A. (2005). Platelet-derived growth factor and reactive oxygen species (ROS) regulate Ras protein levels in primary human fibroblasts via ERK1/2. Amplification of ROS and Ras in systemic sclerosis fibroblasts. *J. Biol. Chem.* 280, 36474-36482.
448. Taipale,J., Miyazono,K., Heldin,C.H., and Keski-Oja,J. (1994). Latent transforming growth factor-beta 1 associates to fibroblast extracellular matrix via latent TGF-beta binding protein. *J. Cell Biol.* 124, 171-181.
449. Takebayashi-Suzuki,K., Pauliks,L.B., Eltsefon,Y., and Mikawa,T. (2001). Purkinje fibers of the avian heart express a myogenic transcription factor program distinct from cardiac and skeletal muscle. *Dev. Biol.* 234, 390-401.
450. Tamaki,T., Ohnishi,K., Hartl,C., LeRoy,E.C., and Trojanowska,M. (1995). Characterization of a GC-rich region containing Sp1 binding site(s) as a constitutive responsive element of the alpha 2(I) collagen gene in human fibroblasts. *J. Biol. Chem.* 270, 4299-4304.
451. Tan,F.K., Hildebrand,B.A., Lester,M.S., Stivers,D.N., Pounds,S., Zhou,X., Wallis,D.D., Milewicz,D.M., Reveille,J.D., Mayes,M.D., Jin,L., and Arnett,F.C., Jr. (2005). Classification analysis of the transcriptome of nonlesional cultured

- dermal fibroblasts from systemic sclerosis patients with early disease. *Arthritis Rheum.* 52, 865-876.
452. Tan,F.K., Stivers,D.N., Foster,M.W., Chakraborty,R., Howard,R.F., Milewicz,D.M., and Arnett,F.C. (1998). Association of microsatellite markers near the fibrillin 1 gene on human chromosome 15q with scleroderma in a Native American population. *Arthritis Rheum.* 41, 1729-1737.
 453. Tan,F.K., Wang,N., Kuwana,M., Chakraborty,R., Bona,C.A., Milewicz,D.M., and Arnett,F.C. (2001). Association of fibrillin 1 single-nucleotide polymorphism haplotypes with systemic sclerosis in Choctaw and Japanese populations. *Arthritis Rheum.* 44, 893-901.
 454. Tantivejkul,K., Loberg,R.D., Mawocha,S.C., Day,L.L., John,L.S., Pienta,B.A., Rubin,M.A., and Pienta,K.J. (2005). PAR1-mediated NFkappaB activation promotes survival of prostate cancer cells through a Bcl-xL-dependent mechanism. *J. Cell Biochem.* 96, 641-652.
 455. ten Dijke,P., Yamashita,H., Ichijo,H., Franzen,P., Laiho,M., Miyazono,K., and Heldin,C.H. (1994). Characterization of type I receptors for transforming growth factor-beta and activin. *Science* 264, 101-104.
 456. Toubi,E., Kessel,A., Grushko,G., Sabo,E., Rozenbaum,M., and Rosner,I. (2002). The association of serum matrix metalloproteinases and their tissue inhibitor levels with scleroderma disease severity. *Clin. Exp. Rheumatol.* 20, 221-224.
 457. Tourkina,E., Gooz,P., Pannu,J., Bonner,M., Scholz,D., Hacker,S., Silver,R.M., Trojanowska,M., and Hoffman,S. (2005). Opposing effects of protein kinase Calpha and protein kinase Cepsilon on collagen expression by human lung fibroblasts are mediated via MEK/ERK and caveolin-1 signaling. *J. Biol. Chem.* 280, 13879-13887.
 458. Trojanowska,M., Wu,L.T., and LeRoy,E.C. (1988). Elevated expression of c-myc proto-oncogene in scleroderma fibroblasts. *Oncogene* 3, 477-481.
 459. Tsukazaki,T., Chiang,T.A., Davison,A.F., Attisano,L., and Wrana,J.L. (1998). SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. *Cell* 95, 779-791.
 460. Turner,J. and Crossley,M. (1999). Mammalian Kruppel-like transcription factors: more than just a pretty finger. *Trends Biochem. Sci.* 24, 236-240.
 461. Unnikrishnan,M. and Burleigh,B.A. (2004). Inhibition of host connective tissue growth factor expression: a novel Trypanosoma cruzi-mediated response. *FASEB J.* 18, 1625-1635.
 462. Utsugi,M., Dobashi,K., Ishizuka,T., Masubuchi,K., Shimizu,Y., Nakazawa,T., and Mori,M. (2003). C-Jun-NH2-terminal kinase mediates expression of connective tissue growth factor induced by transforming growth factor-beta1 in human lung fibroblasts. *Am. J. Respir. Cell Mol. Biol.* 28, 754-761.
 463. Van Beek,J.P., Kennedy,L., Rockel,J.S., Bernier,S.M., and Leask,A. (2006). The induction of CCN2 by TGFbeta1 involves Ets-1. *Arthritis Res. Ther.* 8, R36.

464. van de Water J., Gershwin, M.E., Abplanalp, H., Wick, G., and von der Mark K. (1984). Serial observations and definition of mononuclear cell infiltrates in avian scleroderma, an inherited fibrotic disease of chickens. *Arthritis Rheum.* 27, 807-815.
465. van der Slot, A.J., Zuurmond, A.M., Bardoel, A.F., Wijmenga, C., Pruijs, H.E., Sillence, D.O., Brinckmann, J., Abraham, D.J., Black, C.M., Verzijl, N., DeGroot, J., Hanemaaijer, R., TeKoppele, J.M., Huizinga, T.W., and Bank, R.A. (2003). Identification of PLOD2 as telopeptide lysyl hydroxylase, an important enzyme in fibrosis. *J Biol. Chem.* 278, 40967-40972.
466. van Setten, G.B., Blalock, T.D., Grotendorst, G., and Schultz, G.S. (2003). Detection of connective tissue growth factor (CTGF) in human tear fluid: preliminary results. *Acta Ophthalmol. Scand.* 81, 51-53.
467. Varga, J. (2003). Endothelin-I receptor antagonist for the treatment of pulmonary arterial hypertension in systemic sclerosis. *Curr. Rheumatol. Rep.* 5, 145-146.
468. Verrecchia, F., Chu, M.L., and Mauviel, A. (2001a). Identification of novel TGF-beta /Smad gene targets in dermal fibroblasts using a combined cDNA microarray/promoter transactivation approach. *J. Biol. Chem.* 276, 17058-17062.
469. Verrecchia, F., Rossert, J., and Mauviel, A. (2001b). Blocking sp1 transcription factor broadly inhibits extracellular matrix gene expression in vitro and in vivo: implications for the treatment of tissue fibrosis. *J. Invest Dermatol.* 116, 755-763.
470. Verrecchia, F., Wagner, E.F., and Mauviel, A. (2002). Distinct involvement of the Jun-N-terminal kinase and NF-kappaB pathways in the repression of the human COL1A2 gene by TNF-alpha. *EMBO Rep.* 3, 1069-1074.
471. Visse, R. and Nagase, H. (2003). Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ. Res.* 92, 827-839.
472. Vorwerk, P., Wex, H., Hohmann, B., Oh, Y., Rosenfeld, R.G., and Mittler, U. (2000). CTGF (IGFBP-rP2) is specifically expressed in malignant lymphoblasts of patients with acute lymphoblastic leukaemia (ALL). *Br. J Cancer* 83, 756-760.
473. Vuorio, T., Kahari, V.M., Black, C., and Vuorio, E. (1991). Expression of osteonectin, decorin, and transforming growth factor-beta 1 genes in fibroblasts cultured from patients with systemic sclerosis and morphea. *J Rheumatol.* 18, 247-251.
474. Wagner, K.R. (2005). Muscle regeneration through myostatin inhibition. *Curr. Opin. Rheumatol.* 17, 720-724.
475. Wahab, N.A., Brinkman, H., and Mason, R.M. (2001a). Uptake and intracellular transport of the connective tissue growth factor: a potential mode of action. *Biochem. J.* 359, 89-97.

476. Wahab,N.A., Weston,B.S., and Mason,R.M. (2005b). Modulation of the TGFbeta/Smad signaling pathway in mesangial cells by CTGF/CCN2. *Exp. Cell Res.* 307, 305-314.
477. Wahab,N.A., Weston,B.S., and Mason,R.M. (2005a). Connective tissue growth factor CCN2 interacts with and activates the tyrosine kinase receptor TrkA. *J. Am. Soc. Nephrol.* 16, 340-351.
478. Wahab,N.A., Yevdokimova,N., Weston,B.S., Roberts,T., Li,X.J., Brinkman,H., and Mason,R.M. (2001b). Role of connective tissue growth factor in the pathogenesis of diabetic nephropathy. *Biochem. J.* 359, 77-87.
479. Wang,J.F., Olson,M.E., Ball,D.K., Brigstock,D.R., and Hart,D.A. (2003). Recombinant connective tissue growth factor modulates porcine skin fibroblast gene expression. *Wound. Repair Regen.* 11, 220-229.
480. Wang,J.F., Olson,M.E., Ma,L., Brigstock,D.R., and Hart,D.A. (2004). Connective tissue growth factor siRNA modulates mRNA levels for a subset of molecules in normal and TGF-beta 1-stimulated porcine skin fibroblasts. *Wound. Repair Regen.* 12, 205-216.
481. Wang,S., deNichilo,M., Brubaker,C., and Hirschberg,R. (2001). Connective tissue growth factor in tubulointerstitial injury of diabetic nephropathy. *Kidney Int.* 60, 96-105.
482. Wang,Z., Zhang,Y., Lu,J., Sun,S., and Ravid,K. (1999). Mpl ligand enhances the transcription of the cyclin D3 gene: a potential role for Sp1 transcription factor. *Blood* 93, 4208-4221.
483. Wenger,C., Ellenrieder,V., Alber,B., Lacher,U., Menke,A., Hameister,H., Wilda,M., Iwamura,T., Beger,H.G., Adler,G., and Gress,T.M. (1999). Expression and differential regulation of connective tissue growth factor in pancreatic cancer cells. *Oncogene* 18, 1073-1080.
484. Weston,B.S., Wahab,N.A., and Mason,R.M. (2003). CTGF mediates TGF-beta-induced fibronectin matrix deposition by upregulating active alpha5beta1 integrin in human mesangial cells. *J. Am. Soc. Nephrol.* 14, 601-610.
485. Whitfield,M.L., Finlay,D.R., Murray,J.I., Troyanskaya,O.G., Chi,J.T., Pergamenschikov,A., McCalmont,T.H., Brown,P.O., Botstein,D., and Connolly,M.K. (2003). Systemic and cell type-specific gene expression patterns in scleroderma skin. *Proc. Natl. Acad. Sci. U. S. A* 100, 12319-12324.
486. Wigley,F.M. (1996). Raynaud's phenomenon and other features of scleroderma, including pulmonary hypertension. *Curr. Opin. Rheumatol.* 8, 561-568.
487. Wigley,F.M., Seibold,J.R., Wise,R.A., McCloskey,D.A., and Dole,W.P. (1992a). Intravenous iloprost treatment of Raynaud's phenomenon and ischemic ulcers secondary to systemic sclerosis. *J Rheumatol.* 19, 1407-1414.
488. Wigley,F.M., Wise,R.A., Miller,R., Needleman,B.W., and Spence,R.J. (1992b). Anticentromere antibody as a predictor of digital ischemic loss in patients with systemic sclerosis. *Arthritis Rheum.* 35, 688-693.

489. Willimann, T.E. and Trueb, B. (1994). Identification of functional elements and reconstitution of the alpha 1(VI) collagen promoter. *J. Biol. Chem.* 269, 332-338.
490. Wong, C., Rougier-Chapman, E.M., Frederick, J.P., Datto, M.B., Liberati, N.T., Li, J.M., and Wang, X.F. (1999). Smad3-Smad4 and AP-1 complexes synergize in transcriptional activation of the c-Jun promoter by transforming growth factor beta. *Mol. Cell Biol.* 19, 1821-1830.
491. Worda, M., Sgonc, R., Dietrich, H., Niederegger, H., Sundick, R.S., Gershwin, M.E., and Wick, G. (2003). In vivo analysis of the apoptosis-inducing effect of anti-endothelial cell antibodies in systemic sclerosis by the chorionallantoic membrane assay. *Arthritis Rheum.* 48, 2605-2614.
492. Workalemahu, G., Foerster, M., Kroegel, C., and Braun, R.K. (2003). Human gamma delta-T lymphocytes express and synthesize connective tissue growth factor: effect of IL-15 and TGF-beta 1 and comparison with alpha beta-T lymphocytes. *J. Immunol.* 170, 153-157.
493. Wunderlich, K., Pech, M., Eberle, A.N., Mihatsch, M., Flammer, J., and Meyer, P. (2000). Expression of connective tissue growth factor (CTGF) mRNA in plaques of human anterior subcapsular cataracts and membranes of posterior capsule opacification. *Curr. Eye Res.* 21, 627-636.
494. Xiao, Z., Liu, X., and Lodish, H.F. (2000). Importin beta mediates nuclear translocation of Smad 3. *J Biol. Chem.* 275, 23425-23428.
495. Xie, S., Sukkar, M.B., Issa, R., Oltmanns, U., Nicholson, A.G., and Chung, K.F. (2005). Regulation of TGF-beta 1-induced connective tissue growth factor expression in airway smooth muscle cells. *Am. J. Physiol Lung Cell Mol. Physiol* 288, L68-L76.
496. Xu, S., Denton, C.P., Holmes, A., Dashwood, M.R., Abraham, D.J., and Black, C.M. (1998a). Endothelins: effect on matrix biosynthesis and proliferation in normal and scleroderma fibroblasts. *J. Cardiovasc. Pharmacol.* 31 Suppl 1, S360-S363.
497. Xu, S.W., Denton, C.P., Dashwood, M.R., Abraham, D.J., and Black, C.M. (1998b). Endothelin-1 regulation of intercellular adhesion molecule-1 expression in normal and sclerodermal fibroblasts. *J. Cardiovasc. Pharmacol.* 31 Suppl 1, S545-S547.
498. Xu, S.W., Howat, S.L., Renzoni, E.A., Holmes, A., Pearson, J.D., Dashwood, M.R., Bou-Gharios, G., Denton, C.P., du Bois, R.M., Black, C.M., Leask, A., and Abraham, D.J. (2004). Endothelin-1 induces expression of matrix-associated genes in lung fibroblasts through MEK/ERK. *J. Biol. Chem.* 279, 23098-23103.
499. Yamamoto, T., Takagawa, S., Katayama, I., and Nishioka, K. (1999a). Anti-sclerotic effect of transforming growth factor-beta antibody in a mouse model of bleomycin-induced scleroderma. *Clin. Immunol.* 92, 6-13.
500. Yamamoto, T., Takagawa, S., Katayama, I., Yamazaki, K., Hamazaki, Y., Shinkai, H., and Nishioka, K. (1999b). Animal model of sclerotic skin. I: Local

- injections of bleomycin induce sclerotic skin mimicking scleroderma. *J. Invest Dermatol.* 112, 456-462.
501. Yamane,K., Ihn,H., Kubo,M., and Tamaki,K. (2002). Increased transcriptional activities of transforming growth factor beta receptors in scleroderma fibroblasts. *Arthritis Rheum.* 46, 2421-2428.
 502. Yamashita,H., ten Dijke,P., Franzen,P., Miyazono,K., and Heldin,C.H. (1994). Formation of hetero-oligomeric complexes of type I and type II receptors for transforming growth factor-beta. *J. Biol. Chem.* 269, 20172-20178.
 503. Yang,D.H., Kim,H.S., Wilson,E.M., Rosenfeld,R.G., and Oh,Y. (1998). Identification of glycosylated 38-kDa connective tissue growth factor (IGFBP-related protein 2) and proteolytic fragments in human biological fluids, and up-regulation of IGFBP-rP2 expression by TGF-beta in Hs578T human breast cancer cells. *J. Clin. Endocrinol. Metab* 83, 2593-2596.
 504. Yang,F., Tuxhorn,J.A., Ressler,S.J., McAlhany,S.J., Dang,T.D., and Rowley,D.R. (2005). Stromal expression of connective tissue growth factor promotes angiogenesis and prostate cancer tumorigenesis. *Cancer Res.* 65, 8887-8895.
 505. Yang,M., Huang,H., Li,J., Li,D., and Wang,H. (2004). Tyrosine phosphorylation of the LDL receptor-related protein (LRP) and activation of the ERK pathway are required for connective tissue growth factor to potentiate myofibroblast differentiation. *FASEB J.* 18, 1920-1921.
 506. Yang,X., Letterio,J.J., Lechleider,R.J., Chen,L., Hayman,R., Gu,H., Roberts,A.B., and Deng,C. (1999). Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. *EMBO J.* 18, 1280-1291.
 507. Yang,Y.C., Piek,E., Zavadil,J., Liang,D., Xie,D., Heyer,J., Pavlidis,P., Kucherlapati,R., Roberts,A.B., and Bottinger,E.P. (2003). Hierarchical model of gene regulation by transforming growth factor beta. *Proc. Natl. Acad. Sci. U. S. A* 100, 10269-10274.
 508. Yazawa,N., Kikuchi,K., Ihn,H., Fujimoto,M., Kubo,M., Tamaki,T., and Tamaki,K. (2000). Serum levels of tissue inhibitor of metalloproteinases 2 in patients with systemic sclerosis. *J Am Acad. Dermatol.* 42, 70-75.
 509. Yin,W., Fang,J., Smiley,E., and Bonadio,J. (1998). 8-Cysteine TGF-BP structural motifs are the site of covalent binding between mouse LTBP-3, LTBP-2, and latent TGF-beta 1. *Biochim. Biophys. Acta* 1383, 340-350.
 510. Yokoi,H., Mukoyama,M., Nagae,T., Mori,K., Suganami,T., Sawai,K., Yoshioka,T., Koshikawa,M., Nishida,T., Takigawa,M., Sugawara,A., and Nakao,K. (2004). Reduction in connective tissue growth factor by antisense treatment ameliorates renal tubulointerstitial fibrosis. *J. Am. Soc. Nephrol.* 15, 1430-1440.
 511. Yokoi,H., Sugawara,A., Mukoyama,M., Mori,K., Makino,H., Suganami,T., Nagae,T., Yahata,K., Fujinaga,Y., Tanaka,I., and Nakao,K. (2001). Role of connective tissue growth factor in profibrotic action of transforming growth

- factor-beta: a potential target for preventing renal fibrosis. *Am. J. Kidney Dis.* 38, S134-S138.
512. Yosimichi,G., Kubota,S., Nishida,T., Kondo,S., Yanagita,T., Nakao,K., Takano-Yamamoto,T., and Takigawa,M. (2006). Roles of PKC, PI3K and JNK in multiple transduction of CCN2/CTGF signals in chondrocytes. *Bone.*
 513. Yosimichi,G., Nakanishi,T., Nishida,T., Hattori,T., Takano-Yamamoto,T., and Takigawa,M. (2001). CTGF/Hcs24 induces chondrocyte differentiation through a p38 mitogen-activated protein kinase (p38MAPK), and proliferation through a p44/42 MAPK/extracellular-signal regulated kinase (ERK). *Eur. J. Biochem.* 268, 6058-6065.
 514. Young-Min,S.A., Beeton,C., Laughton,R., Plumpton,T., Bartram,S., Murphy,G., Black,C., and Cawston,T.E. (2001). Serum TIMP-1, TIMP-2, and MMP-1 in patients with systemic sclerosis, primary Raynaud's phenomenon, and in normal controls. *Ann. Rheum. Dis.* 60, 846-851.
 515. Yu,B., Datta,P.K., and Bagchi,S. (2003a). Stability of the Sp3-DNA complex is promoter-specific: Sp3 efficiently competes with Sp1 for binding to promoters containing multiple Sp-sites. *Nucleic Acids Res.* 31, 5368-5376.
 516. Yu,L., Border,W.A., Huang,Y., and Noble,N.A. (2003b). TGF-beta isoforms in renal fibrogenesis. *Kidney Int.* 64, 844-856.
 517. Yue,J., Sun,B., Liu,G., and Mulder,K.M. (2004). Requirement of TGF-beta receptor-dependent activation of c-Jun N-terminal kinases (JNKs)/stress-activated protein kinases (Sapks) for TGF-beta up-regulation of the urokinase-type plasminogen activator receptor. *J Cell Physiol* 199, 284-292.
 518. Zawel,L., Dai,J.L., Buckhaults,P., Zhou,S., Kinzler,K.W., Vogelstein,B., and Kern,S.E. (1998). Human Smad3 and Smad4 are sequence-specific transcription activators. *Mol. Cell* 1, 611-617.
 519. Zeisberg,M., Hanai,J., Sugimoto,H., Mammoto,T., Charytan,D., Strutz,F., and Kalluri,R. (2003). BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. *Nat. Med.* 9, 964-968.
 520. Zhang,W., Ou,J., Inagaki,Y., Greenwel,P., and Ramirez,F. (2000). Synergistic cooperation between Sp1 and Smad3/Smad4 mediates transforming growth factor beta1 stimulation of alpha 2(I)-collagen (COL1A2) transcription. *J. Biol. Chem.* 275, 39237-39245.
 521. Zhang,Y., Feng,X., We,R., and Derynck,R. (1996). Receptor-associated Mad homologues synergize as effectors of the TGF-beta response. *Nature* 383, 168-172.
 522. Zhang,Y. and Gilliam,A.C. (2002). Animal models for scleroderma: an update. *Curr. Rheumatol. Rep.* 4, 150-162.
 523. Zhang,Y., McCormick,L.L., Desai,S.R., Wu,C., and Gilliam,A.C. (2002). Murine sclerodermatous graft-versus-host disease, a model for human scleroderma: cutaneous cytokines, chemokines, and immune cell activation. *J. Immunol.* 168, 3088-3098.

524. Zhang, Y., McCormick, L.L., and Gilliam, A.C. (2003). Latency-associated peptide prevents skin fibrosis in murine sclerodermatous graft-versus-host disease, a model for human scleroderma. *J. Invest Dermatol.* 121, 713-719.
525. Zhou, X., Tan, F.K., Milewicz, D.M., Guo, X., Bona, C.A., and Arnett, F.C. (2005a). Autoantibodies to fibrillin-1 activate normal human fibroblasts in culture through the TGF-beta pathway to recapitulate the "scleroderma phenotype". *J Immunol.* 175, 4555-4560.
526. Zhou, X., Tan, F.K., Wang, N., Xiong, M., Maghidman, S., Reveille, J.D., Milewicz, D.M., Chakraborty, R., and Arnett, F.C. (2003). Genome-wide association study for regions of systemic sclerosis susceptibility in a Choctaw Indian population with high disease prevalence. *Arthritis Rheum.* 48, 2585-2592.
527. Zhou, X., Tan, F.K., Xiong, M., Arnett, F.C., and Feghali-Bostwick, C.A. (2005b). Monozygotic twins clinically discordant for scleroderma show concordance for fibroblast gene expression profiles. *Arthritis Rheum.* 52, 3305-3314.
528. Zhou, X., Tan, F.K., Xiong, M., Milewicz, D.M., Feghali, C.A., Fritzler, M.J., Reveille, J.D., and Arnett, F.C. (2001). Systemic sclerosis (scleroderma): specific autoantigen genes are selectively overexpressed in scleroderma fibroblasts. *J. Immunol.* 167, 7126-7133.
529. Zojer, N., Keck, A.V., and Pecherstorfer, M. (1999). Comparative tolerability of drug therapies for hypercalcaemia of malignancy. *Drug Saf* 21, 389-406.

Appendix I

Criteria for the classification of Systemic Sclerosis

Criteria	Characteristic	Definition
A. <i>Major criterion</i>	Proximal	Symmetric thickening, tightening, and induration of the skin of the fingers and the skin proximal to the metacarpophalangeal or metatarsophalangeal joints. The changes may affect the entire extremity, face, neck, and trunk.
	Scleroderma	
B. <i>Minor criterion</i>	1. Sclerodactyly	Above-indicated changes limited to the fingers.
	2. Digital pitting scars	Depressed areas at tips of fingers or loss of digital pad tissues as a result of ischaemia.
	3. Bibasilar pulmonary fibrosis.	Bilateral reticular pattern of linear or lineo-nodular densities most pronounced in basilar portions of the lungs on standard chest roentgenogram; may assume appearance of diffuse mottling or honeycomb lung. These changes should not be attributable to primary lung disease.

A patient is classified as having SSc if one major or two or more minor criteria are present. Modified from Black et al 1998 (**Black and Denton, 1998b**)

Appendix II

CTGF promoter sequence

A

ACTAGT^{SpeI}CCTTTCAAAATATTTATCCTTTCACTTACTTCTATTATTTGAGATAAGA
TAGCTAGTCCCAATTCTCTCTTCCTCCTAAACCTATTGATTGGGTGTCCCCAAATCAC
ACATCTTAAGAAATGCTTATTTCTCTGTTTTTTCATTCCCCTCACTTAAATATCACC
TTAAGTATCACCAAGGGGATGCTTCACATTTAGCCCTGATGAAGCAAGGGGCTGTAA
TACTCCTGGTTGCTAAACGGTGATGAATTTGAGATTCTAACCTGTGAGGACAGTGTA
AGAGACCACCAGGCTCATTCTACCTAGCAGCATCCTCATCTGACACTATCTTAGATG
AACAAAAGCTGACTAGAGGCAACACAGGATTCTAAGTAACTACTTTAAAAAAAAA
GAGTCAAATTAACAAAATAAGTGAAACAAACAGTAAGTTGAAGGGATATCATCTTACA
AAACAAAACCTTCTTTCTCACCCTCTGGCCTCCAACCTCTATGGACAAGCGAGGAACA
GGCAAACATAAAAACACAACACATGCAACGGCCTTGACTGCCTTCTGACAAAATACCT
TTGCATTAGAAAAATCTCTAAGAAAGT**CAGAGAAC**TAATTATAAGGAGGAAAAGGCAT
GAAGGTAATGAATGTAATTTAGATAGTAAACATCCGCGTTTATCTTCCCCGCCATGAA
CTCCCAGCAAGTACGCCCTCTCCTTAACCTTCATCTTAAGCAAACAGGAGGTCATGTC
AGCCAAACCAGGGTCCCTGAAGATTTTGAATAATATTTATTACTATTTTACAAAAGG
CTTCTGGGATACCATTTTTTTTTTGTAGAATTAATTTACATGATTGAATTTGAGGCACG
CTAGAAACATCAGAAACCTCCCCCTGACCCACCATAAGGACAAATCGCTCACTGTCCA
GATGCCCATATACGGCCAGACTTCCTCCTATAAGTCTGACAGCTCCGTGTATGCATTG
CGTATGGGTCTTGGCTCTGAGTCACATTCCTCCGGCATGTTTCCCTGGGAGCTTTGAT
TTCCGCTTTGAGGGCTGGCACTGACTCCTACTTGCTCTTATAAGAAAATGTCTACAAC
TAAAGTTGAAAATGTGCAAGATTCTTCTTTAAGTACACAAACACTCCAACCTCTGGC
CACTTTTCTGTAGTCCTGCCAGTTTTCTCTGTGTCTGATACCTGATCAAGAAACCTT
TTTTTTTTAGGGAGGAAAAACAACAAAAAATTAAGGGAAGGAATGTAATACATCCA
CAGCTGAACATCTTACAAGACTTAACCAAACGACCAGTACAAATGATGCCCTAGTAGA
CACACACACATACCTTATATAGCTTGGTAAACAGGACTCAGTGGCCAGCTTAGTTAAT
TCCTCATTTTACCACGAGGAAAAAATGCTCTCTAAAAAGCATTTGCTAGATAAATACT
TGCTTACAAGTCAAAATTAGTGCATCTTAACCATTTTGACACAAATTCAGATCTT
TTTCTCCCACTCTGACCTGCTGTCAATCAAAGGGGCTGGGGTAAGTCAAAGTTGGAT
GAAGTTATAAGAAGCCACTATTTCCCCTTTACCGCCCAGGCACACACAAACATTTGC
TGAGTCATTGATAATAGTGCAAGAATTCAGATCCCATGGCATGAATTATGGATGGAGT
ATCAGCCTTTCCCTTTGAGTTTTCTGGCATTGATGGTCCATATCACAATAAAAACAT
ACTTCTGAGTCTGTGAATGTCTTGGGTGTCTTCTCTCATCTCTCTCTCTCTCTCTCT
GTGTGCATGTATGTCTGTCTGTCTGTCTGTCTGTCTGTCTCTTAATAATAGTCTTTTAAC
TCCTTCAAAAAGAGTTTTTGATCATAAAGCCTCAATCTAACCTGATAATGCACAGAAAC
TTTTTCTTTCTGAGATTTTCATATGTTCTATATATTTCAATATGCTTATTTCTCTTA
AGCAACATTAAAATCATCTATTTGGAAGTACTATAGATTTTTTTGAGACTTCGAGGG
ACTATGAGAATTTTATAGTAAAACCAAATCTTTCTTTTGACATTTTTTAACAA**CAGAG**
CAGAATGGTAATATACTTATTATACACAGAGATTGAAAAAAATCAAGAAAATCTGAA
AAAAATAAAGCACCAATTCCTGGAATTTTCATCTACTCAGCCCTCACTGAACCCTAT
TAAATTAAAAAATTATGATTACTTTTCATTTTTTAAACAGAAATGGGAATATGGCTCC
TACGTGTGACTTCCCTGAGATCTGCGAAAGGCAGTTCCAAGAATACAACCTAGGCCAT
CTAACTATAACCCAGTACAACAATTTATCCCTCCATGAATGAGTCTCCCTGCTGCAG
GCATGAAGATGGTGGTAGCTTTTCACTCTCCTGAGGATTCAGAAATCCCCAACCTT
TCTCCACCTACCTCACTCTGCAAGCTAGGAGAGCTCACCACCAAGCCACATCCAC
CTTAGGTGAGACTCGCACTGCACATCCCTACCCTTCCCCAGCCACCACACCTTTCCAA
CCATTAGAGGTAGATGTAATTGGGGGTTTAGGTCTTGAAAGGTTTCACTGTGGCTTCC
CACATATTTTGGACTTTTAAATCATCCACAAGGGAAGAAAAAGTTGAGGGCCAAGTT
GCCTTTAATACACATGTGGCTATTAAGTTAACTTGAATGTTCTTCTTTAAATAGGA
ACTCTTAGGCCTCTTCTTTTTGAGGAATGCTTGGAAAGGACATTAAATAACAATGACG
AAAATTCTTCTGAGCTT**CAGAGGGC**ACCCCCACCCCTTGTGGCCATTACACTAT
TGACATGCAACTACACTCTATAACATGTGGCTGGCTTAACGAGGTAATCGTGCACAGA

TGATATGCTCTATGCTGAAGTTGGAGTGGTTATATTTCTTCTTGGCTATTTTTCAACA
 GACTGTACTGAACTTTAAAAATTTGATCAGTTTAAACAGGCTGTTTTCAAATGATAA
 ATCTATAATGCATTGTTTCTAAAGCCGATCTTTCACCACAAACAGATATGCTTATCT
 TGAACCCATGCTCTGAAGCATCAACAACCTTAAAGGAATCTACTCATTGGTAACACCAA
 TGTCAAATGATGCGGCATTACAGAATACAAAAATGGTATGGCCCTAGAATCACTCTCT
 TTATATAAAATGGGAACTGGAGGTATTAACAAAAAGCCACTAAACAACATAGATTCC
 AAATGAAAGCACAGTGAATTAGTTGGGTCTTTAGTGAAGCTAAGTAGCACTATATTAA
 GAAATGGAGATATGCTTAGGTTTATTGGCCATATTCAACATCTGCACACACCCAGGGA
 GAATAAAGCCTATTCTTTCCTTGTCTGACCTCACTCCCTCAGGCTGCATGTTCTCTG
 GGTAATGAGAAGTCACAATCACTATTATAGATGTGTGGGGAGTCACTAAAAATATAT
 TATTCAGTGTCAATCTTAGTTTATATCCAGATACAACAGGGTACACTGCTCTTGTAAT
 GGAATCAGACTTCTTATTTTAAACAAGACAAACCAATCCAATCCACATTTGAAGATTA
 TAGGTTTTTAATATAAGAAAATGCATCTATTTCTCAAAGACCCTAGTGAAGCTGTGTTT
 AAATGCTCCTAGGTGAACCCCTTTTGCATCCCAGTGTTCACCCCTGACACCCAGAGC
 CCCTACCTACCCAACACAGAATCATTTGCTCTGATAGAACAAATGGATCCCTTTTTTCTG
 GAAACATTGATGGCCACTCCTCCCTTGTCCTTGCCTATATAAACTCCTACATATATT
 AAGAGAAAATAAGCAAGAGTTTTTGAAATCTGCCCCAGGAGACTGCATCCTGAGTCA
 CACGCGTCTTTGTTCTCTTTCTTGTCCCAAAACCGTTACCTCAAGTGACAAATGATCA
 AATCTCAAATATAGAATTCAGGGTTTTACAGGTAGGCATCTTGAGGATTTCAAATGGT
 TAAAGCAACTCACTCCTTTTCTACTCTTTGGAGAGTTTCAAGAGCCTATAGCCTCTA
 AAACGCAAATCATTGCTAAGGGTTGGGGGGGAGAAACCTTTTCGAATTTTTTAGGAAT
 TCCTGCTGTTTGCCTCTTCAGCTACCTACTTCTTAAAGGATGTATGTCAGTGGACA
 GAACAGGGCAAACCTATTTCGAAAAAGAAATAAGAAATAATTGCCAGTGTGTTTATAAA
 TGATATGAATCAGGAGTGGTGCGAAGAGGATAGGGAAAAAAAAAATTCTATTTGGTGCT
 GGAAATACTGCGCTTTTTTTTTTCTTTTTTTTTTTTCTGTGAGCTGGAGTGTGCCA

(1) 5'-GAGCTGGAGTGTGCCA

GCTTTTTCAGACGGAGGGAATGCTGAGTGTCAAGGGGTGAGGATCAATCCGGTGTGAGT
 GCTTTTTGGATCCGGAGGAATGCTGAGTGTC-3'

(2) 5'-CAGACGGAGGAATGCTGGGGATCCCGAGGATCAATCC-3'

TGATGAGGCAGGAAGGTGGGGAGGAATGCGAGGAATGTCCTGTTTGTGTAGGACTCC
 ATTCAGCTCATTGGCGAGCCGCGGCCGC^{NotI}CCGAGCGTATAAAAGCCTCGGGCGGCG

5'-CACGTCATTGGCGAGCCGCGATATC CCGAGCGTATAAAAGCCTC-3' (3)

5'-GTATAAAAGCCTCGGGCCGCG

CGCCCGAACT⁺¹CACACAACAACCTCTCCCCGCTGAGAGGAGACAGCCAGTGCGACTC
GCCGCCAACT CACACAACAAC-3' (4)

CACCTCCCTCGAG^{XhoI}

B

Sequence	Transcription factor binding site
CAGACGGA	Smad3 responsive element
GTGTCAAGGGGTC	BCE-1 element
GAGGAATGCGAGGAAT	TEF elements
GGAA	ETS binding sites
CCGCCCGCCCC	Sp-binding site
CAGAGGGC	Putative Smad binding sites
TATAAAA	TATA Box
GCCACTC (C/G) TCCC	Polymorphism linked to SSc (Fonseca <i>et al.</i> in preparation)
AACT ⁺¹ CAC	The position of all transcription factor binding sites are defined relative to those previously defined (Grotendorst <i>et al.</i> , 1996) and are 199 bp up stream to ATG transcription start site (Accession number: GI:49456426)

CTGF promoter sequence: Restriction enzyme sites used in generating pCTGF4.5 are as indicated: SpeI (ACTAGT); NotI (GCGGCCGC) XhoI (CTCGAG) are as indicated. CTGF promoter deletion series and point mutations were kindly generated in collaboration with Dr Andrew Leask and constructed, as defined in Chapter 3, Section 3.3.1.3. CTGF promoter deletion series were constructed by PCR using oligonucleotide primers targeted to sequences in the CTGF promoter (-805, -244, -166, -86 and +17) as indicated by italics. Generation of CTGF promoter reporter constructs (1) CTGFΔSMAD; (2) CTGFΔTRE (CTGFΔBCE-1); (3) 5'Sp1 CTGF; (4) 3'Sp1- using mutagenic oligonucleotides are shown in grey. Point mutations introduced in the generation of these constructs are underlined (A). Relevant identified and putative transcription factor binding sites and their discription are shown (B).

Appendix III

Publications arising from this thesis

THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 276, No. 14, Issue of April 6, pp. 10594–10601, 2001
Printed in U.S.A.

CTGF and SMADs, Maintenance of Scleroderma Phenotype Is Independent of SMAD Signaling*

Received for publication, November 7, 2000, and in revised form, December 11, 2000
Published, JBC Papers in Press, January 4, 2001, DOI 10.1074/jbc.M010149200

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In normal adult fibroblasts, transforming growth factor- β (TGF β) induces the expression of connective tissue growth factor (CTGF). CTGF independently promotes fibroblast proliferation and matrix deposition, and in acute models of fibrosis promotes cell proliferation and collagen deposition acting synergistically with TGF β . In contrast to normal fibroblasts, fibroblasts cultured from fibrotic tissues express high basal levels of CTGF, even in the absence of added TGF β . Induction of transcription by TGF β requires the action of SMAD proteins. In this report we have investigated the role of SMADs in the TGF β -induction of CTGF in normal fibroblasts and in the elevated levels of CTGF expression found in dermal fibroblasts cultured from lesional areas of patients with scleroderma, a progressive fibrotic disorder that can affect all organs of the body. We have identified a functional SMAD binding site in the CTGF promoter. TGF β -induction of CTGF is dependent on SMAD3 and SMAD4 but not SMAD2 and is p300-independent. However, mutation of the SMAD binding site does not reduce the high level of CTGF promoter activity observed in dermal fibroblasts cultured from lesional areas of scleroderma patients. Conversely, the previously termed TGF β RE in the CTGF promoter is required for basal CTGF promoter activity in normal fibroblasts and for the elevated level of CTGF promoter activity in scleroderma fibroblasts. Thus, the maintenance of the fibrotic phenotype in scleroderma fibroblasts, as visualized by excess CTGF expression, appears to be independent of SMAD-dependent TGF β signaling. Furthermore, given CTGF's activities, the high level of CTGF expression observed in scleroderma lesions may contribute to the excessive scarring observed in this disorder.

Wound healing requires the synthesis and reconstitution of properly organized connective tissue. If activation of collagen gene expression persists, uncontrolled connective tissue deposition results, leading to pathologic scarring and fibrosis (1–3) such as in scleroderma (systemic sclerosis), which is characterized by the progressive scarring of skin and certain internal

organs (4). Given the ability of TGF β ¹ to promote fibroblast proliferation and matrix synthesis, attention has been devoted to its potential role in initiating and maintaining the fibrotic phenotype (for reviews, see Refs. 5 and 6), including scleroderma (7). For example, there is a clear correlation between TGF β action and the initiation of fibrosis; in acute drug- or surgery-induced animal models, anti-TGF β strategies are effective at blocking the onset of fibrosis (for review, see Ref. 6). However, the fibrosis is clinically a chronic disorder; the involvement of TGF β in the maintenance of fibrosis and the effectiveness of anti-TGF β strategies in the reversal of fibrosis is unclear.

In terms of scleroderma, the data supporting the role of TGF β in the fibrotic phenotype is circumstantial, chiefly depending on the histological distribution of TGF β mRNA and protein. Unfortunately, the data are often contradictory. For example, mononuclear cells taken from bronchial lavage fluids of scleroderma patients have elevated TGF β levels (8). However, in the actual lesional areas of skin, TGF β mRNA is only localized to the leading edge of the scleroderma lesion; i.e. to the region of enhanced inflammatory response that is presumably involved with the initiation of the fibrotic response (9). Furthermore, fibroblasts taken from scleroderma lesions show elevated levels of collagen relative to their normal counterparts, yet show little difference in their ability to produce TGF β or in their ability to bind TGF β , nor do they show enhanced sensitivity to TGF β treatment (10, 11). Thus, although there seems to be circumstantial data to support the role of TGF β in the onset of the scleroderma fibrotic phenotype, it is unclear as to its precise role in initiating or maintaining the scleroderma phenotype.

In an initial attempt to molecularly characterize the scleroderma phenotype, we recently used differential display analysis to identify genes up-regulated in dermal fibroblasts cultured from patients with scleroderma (12). Perhaps the most interesting gene up-regulated in scleroderma fibroblasts was connective tissue growth factor (CTGF) (12). CTGF is a heparin-binding 38-kDa cysteine-rich peptide that induces proliferation, collagen synthesis, and chemotaxis in mesenchymal cells (13–18) and has been shown to potentiate sustained fibrosis when injected along with TGF β in an acute animal model (19). Previously, CTGF mRNA and protein were shown to be constitutively expressed in numerous fibrotic disorders both in skin and in internal organs, such as atherosclerosis and pulmonary and renal fibrosis, and that this expression correlated with

* This work was supported in part by National Institutes of Health Grant AR45879, Arthritis Research Campaign (UK), The Raynaud's and Scleroderma Association Trust, and the Nightingale Charitable Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: TGF β , transforming growth factor- β ; TGF β RE, TGF β response element; CTGF, connective tissue growth factor; DMEM, Dulbecco's modified Eagle's medium; HA, hemagglutinin; PAI-1, human plasminogen activator inhibitor; SEAP, secreted enhanced alkaline phosphatase.

high collagen synthesis (12, 20–24). Hence, in adult tissues, constitutive CTGF expression is considered a faithful, clinical, molecular marker of fibrosis. Furthermore, given its activity, CTGF is considered a mediator of the fibrotic phenotype (12–24).

In contrast to the situation in fibrotic disorders, CTGF is not expressed in normal human dermal or mouse NIH 3T3 fibroblasts unless cells are treated with TGF β (14–16, 20, 25). Similarly, exogenous addition of TGF β increases the amount of CTGF protein and promoter activity produced by fibroblasts cultured from scleroderma lesions (12, 20). This induction by TGF β is cell-type specific, as it occurs in connective tissue cells but not in epithelial cells or lymphocytes (14–16). The regulation of CTGF expression by TGF β appears to be controlled primarily at the level of transcription (20, 25). Originally, the up-regulation of CTGF by TGF β was thought to be solely dependent on a relatively small sequence present in the 5' upstream region of the CTGF promoter (TGF β response element; TGF β RE) (25). This sequence does not resemble the TGF β response elements described in other genes, including the SMAD recognition sequence (25). However, recently we have shown that sequences immediately upstream of the previously identified TGF β RE are required for TGF β to induce CTGF expression (20). Similarly, the high level of CTGF protein observed in scleroderma appears to be due, at least in part, to gene transcription because the CTGF promoter activity is substantially higher in scleroderma dermal fibroblasts relative to normal dermal fibroblasts (12). However, the precise mechanism underlying the control of CTGF gene expression in normal and fibrotic fibroblasts remains unknown.

Activation of TGF β -dependent gene expression is commonly mediated through SMADs 2, 3, and 4 (for reviews, see Refs. 25 and 26). SMADs 2 and 3 are normally present in the cytosol. Once activated by TGF β , SMADs 2 and 3 interact transiently with type I TGF β receptor kinase and become phosphorylated at their carboxyl terminus (27, 28). SMAD2 and SMAD3 then form a heteromeric complex with SMAD4 (29, 30). These complexes then translocate to the nucleus and activate expression of target genes (25), in concert with other nuclear factors the identity of which can vary depending on the promoter and cell type (e.g., Refs. 31–34). Recent studies have recognized a consensus DNA motif, GTCTAGAC that provides the binding site for the SMAD3-SMAD4 complex (35). Homologs of this element have been identified in the promoters of several TGF β -responsive genes (e.g., Refs. 36–39). More recently, studies in TGF β -responsive systems have also identified other SMAD family members, such as SMADs 6 and 7, which represent a functionally distinct class of SMADs that antagonize SMAD-receptor interactions (40).

In this report, we assess the role of SMADs in CTGF gene expression in normal and scleroderma fibroblasts. Using a combination of Western blot, gel shift, and gene cotransfection/promoter assays, we show that a functional SMAD binding site in the CTGF promoter is necessary for the induction of CTGF by TGF β in fibroblasts. Conversely, the constitutive CTGF expression observed in scleroderma appears to be independent of SMAD action. Thus, the maintenance of the fibrotic phenotype in scleroderma, as visualized by constitutive CTGF expression, appears to be SMAD-independent. In addition, we show that the previously termed TGF β RE is required for basal CTGF expression in normal fibroblasts and elevated CTGF expression in scleroderma.

MATERIALS AND METHODS

Cell Culture, Reporter Assays, and Transfections—NIH 3T3 fibroblasts (ATCC) were maintained in DMEM, 10% calf serum (Life Technologies). Fetal SMAD3-knockout mouse fibroblasts, and their wild-

type counterparts, were a gift from Anita Roberts (National Institutes of Health). Primary dermal fibroblasts from lesional areas of scleroderma patients and normal individuals were taken from biopsies of patients with diffuse cutaneous scleroderma and age, sex, and anatomically site-matched healthy volunteers, respectively. All patients fulfilled the criteria of the American College of Rheumatology for the diagnosis of scleroderma. Informed consent and ethical approval were obtained for all procedures. Fibroblasts were maintained in DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (Life Technologies). All cells were used between passages 2 and 5, with reculture at a dilution of 1 in 3 passaged fibroblasts (41). Transfections were performed in 6-well plates using LipofectAMINE plus, as described by the manufacturer (Life Technologies). For reporter assays, 1 μ g of a reporter construct was cotransfected with 0.25 μ g of CMV β -galactosidase plasmid, which was used as an internal transfection control (CLONTECH). For assays in which the role of transacting proteins was to be tested, 0.5 μ g of reporter, and 1 μ g of empty vector or expression vector encoding the protein of interest were used. For transfections involving *shl* and *shn*, amounts of DNA used were as described in the text. SEAP and β -galactosidase kits were used to perform reporter gene assays (Tropix). TGF β 2 was from R&D Systems or Celsix and used at concentrations indicated in the text. The data are presented as mean \pm S.E., or representative of experiments performed in at least triplicate in at least two separate experiments. Statistical analysis was performed by the Student's unpaired *t* test. *p* values less than 0.05 were considered statistically significant.

DNA Constructs—The full-length CTGF promoter/SEAP reporter construct is described elsewhere (20). Point mutations were introduced using a kit (Stratagene). The mutagenic primer (Sigma Genosys) for the SMAD mutant was 5'-GAGCTGGAGTGTCCAGCTTTTGGATCCGG-AGGAATGCTGAGTGTTC-3' and for the TGF β RE mutant was 5'-CAG-ACGGAGGAATGCTGGGGATCCCGAGGATCAATCC-3'. Constructs were fully sequenced in both directions to confirm mutagenesis before use. SMADs 2, 3, 4, 6, and 7 expression vectors were generous gifts from Joan Massagué (Sloan-Kettering), and *shl* and *shn* expression vectors were generous gifts from Robert Weinberg (Whitehead Institute). Expression vectors encoding wild-type and dominant-negative p300 were from Upstate Biotechnology. The human plasminogen activator inhibitor (PAI-1)/luciferase construct was a generous gift from Dan Rifkin (New York University).

Western Blot Analysis—Human dermal fibroblasts were grown in DMEM and 10% fetal bovine serum. Cells were grown to confluence and switched to DMEM supplemented with insulin/transferrin/selenium. Cells were grown for an additional 18 h, and then 10 ng/ml TGF β 2 (R&D Systems) was added. Media was removed 24 h later, and 25 μ l was electrophoresed through a 12% SDS-polyacrylamide gel (Novex) and blotted to nitrocellulose (Bio-Rad). Filters were blocked overnight at 4 °C in 5% nonfat dry milk in Tris-buffered saline, 0.1% Tween 20. CTGF protein was detected by a 1 h incubation of a 1:1000 dilution of rabbit anti-CTGF antibody in 1% milk, TBS, 0.1% Tween 20. For anti-SMADs 2, 3, and 7 Westerns, cells were lysed in 2% SDS, and protein concentration was determined (Bio-Rad). Equal amounts of protein were electrophoresed as described above. For experiments involving SMADs, SMAD proteins were detected using a 1:1000 dilution of antibody. SMADs 3, 4, and 7 antibodies were from Santa Cruz Biotechnology. For experiments involving the detection of transfected FLAG-tagged SMADs 3 and 4, 1:5000 dilution of anti-FLAG antibody (Sigma) was used. For detection of HA-tagged transfected *shl* and *shn*, an anti-HA antibody (Roche Molecular Biochemicals) was used. In all cases, proteins were detected by 1:5000 dilution of an appropriate horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch or Roche Molecular Biochemicals). Proteins were then detected by chemiluminescence (Pierce). Bands on Western blots were quantified using a computer program (AlphaEase, Alpha Innotech), and values were expressed in arbitrary densitometric units.

Electrophoretic Mobility Shift Assays—Protein extracts were prepared from $\sim 1 \times 10^6$ NIH 3T3 control cells or cells treated with TGF β 2 (2 ng/ml) for 60 min following serum starvation for 24 h. Cells were then lysed, and protein nuclear extracts were prepared as previously described (42). Protein concentrations were determined using the Bio-Rad protein assay kit. Oligonucleotide probes as indicated were 32 P-labeled, gel purified, and incubated with nuclear extracts for 30 min at 30°. Gel shift conditions consisted of 1–3 μ l of nuclear extract (containing 2–5 μ g protein) and 0.5 ng probe (1.5×10^4 cpm) in a final volume of 15 μ l of 150 mM NaCl, 10 mM Tris-HCl, pH 7.5 and 50 μ g per ml poly(dI-dC) (Roche Molecular Biochemicals). For antibody treatment, nuclear extracts were incubated with 2 μ g of the indicated antibodies prior to

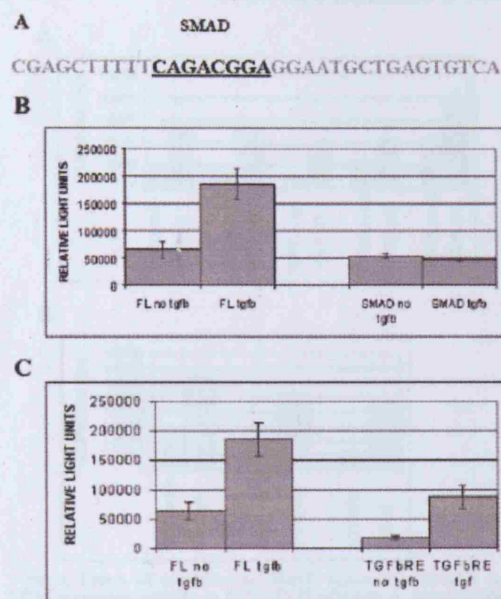


FIG. 1. Effect of mutating a putative SMAD binding site on CTGF expression. **A**, sequence of the CTGF promoter between nucleotides -183 and -150, in gray. The sequence between -244 and -166 has also previously been shown to be necessary for TGF β -induced CTGF expression (Ref. 20). A consensus SMAD binding site located between -173 and -166 is shown underlined and in black. The sequence in gray was made into a double-stranded oligomer for use as a gel shift probe (see Fig. 2). **B** and **C**, in the context of a full-length (805 base pair) promoter construct, the SMAD binding site (**B**) or TGF β RE (Ref. 25, **C**) was mutated to a *Bam*HI site, as described under "Materials and Methods." The resultant constructs (SMAD or TGF β RE, respectively) were transfected into NIH 3T3 fibroblasts, and expression was compared with that of the unmutated wild-type full-length construct (**FL**). Cells were serum-starved for 24 h, followed by an additional 24-h incubation with or without addition of 25 ng/ml TGF β 2 (*tgf* or *no tgf*, respectively). Assays were performed as described under "Materials and Methods." Relative light units based on SEAP expression (25 μ l of conditioned media) normalized to β -galactosidase activity (from a co-transfected CMV- β -galactosidase plasmid) are shown (mean \pm S.E., $n = 18$).

addition of probe. SMAD2 antibody was from Zymed Laboratories Inc. and SMAD3 and SMAD4 antibodies were from Santa Cruz Biotechnology. For competition experiments, 50- or 100-fold excess cold competitor (25 or 50 ng, respectively of unlabeled probe) was added to the reaction mixture before incubation when required. Complexes were resolved on 6% nondenaturing acrylamide gels using 0.5 \times Tris-Borate-EDTA. Gels were then dried, and complexes were visualized by autoradiography.

Immunoprecipitations—NIH 3T3 cells were transfected as described above with 2 μ g each of expression vector encoding SMAD3-FLAG, SMAD4-FLAG, ski-HA or smcNHA per 100-mm dish. Forty-eight hours later, cells were lysed in 800 μ l of ice-cold freshly prepared 50 mM Hepes, 10% glycerol, 0.5% Nonidet P-40, 50 mM NaCl, 1 mM dithiothreitol, 50 mM sodium fluoride, 25 mM β -glycerol phosphate, 1 mM EDTA, 1 mM sodium pervanadate, 2 mM phenylmethylsulfonyl fluoride, pH 7.9. Cells were lysed on ice, scraped, sonicated, and spun. To 100 μ l of lysate, 40 μ l of agarose affinity gel conjugated with an anti-FLAG antibody (Sigma) was added. Gel without antibody was used as a control. Gel and protein slurry was rotated on a shaker for 2 h at 4 $^{\circ}$ C. Beads were washed in lysis buffer (1 ml) three times. Protein was eluted with protein sample buffer (100 μ l) and stored at -80 $^{\circ}$ C. Samples (25 μ l) were then subjected to polyacrylamide gel electrophoresis through a 4–20% gel (Novex), and gels were blotted onto polyvinylidene difluoride membrane (Millipore). After blocking membrane for 2 h at room temperature in phosphate-buffered saline, 5% nonfat dry milk, 0.1% Tween

Protein	-	+	+	+	-	+	-	+	+
Cold probe	-	-	50	100	-	-	-	-	-
Anti-SMAD2	-	-	-	-	+	+	-	-	-
Anti-SMAD3	-	-	-	-	-	-	+	+	-
Anti-SMAD4	-	-	-	-	-	-	-	-	+

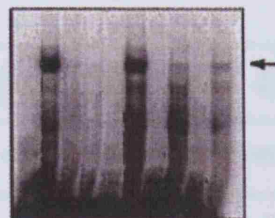


FIG. 2. SMADs 3 and 4 bind the CTGF promoter. A double-stranded oligomer probe (0.5 ng) containing the putative SMAD binding site of the CTGF promoter (shown in Fig. 1) was used in a gel shift with nuclear extracts prepared from NIH 3T3 cells that had been treated for 1 h with TGF β 2. A complex was observed (arrow) that was specific because 50 or 100-fold excess (25 ng and 50 ng, respectively) of cold competitor oligonucleotide could compete for binding, as shown. Anti-SMAD2, 3, or 4 (1 μ l) antibodies were preincubated with nuclear extract before addition of probe.

20, blots were incubated overnight at 4 $^{\circ}$ C with a 1:2000 dilution of anti-HA antibody (Covance). After extensive washing, proteins were detected by a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-mouse antibody (Roche Molecular Biochemicals or Jackson ImmunoResearch). Antibodies were diluted in blocking buffer. A chemiluminescent detection kit was used to observe proteins (Pierce).

RESULTS

SMADs Are Necessary for the TGF β -mediated Induction of CTGF in a p300-independent Fashion—Recently, we identified a segment of the CTGF promoter between nucleotides -244 and -166 that was required for the TGF β -mediated induction of CTGF (20). Sequence inspection of the region of the CTGF promoter between -244 and -166 resulted in the identification of a putative consensus SMAD site (35–38) immediately upstream of -166 (Fig. 1A). Mutating this sequence to a *Bam*HI site in an otherwise wild-type, full-length CTGF promoter/SEAP reporter construct resulted in complete abolition of TGF β -induced gene expression when this construct was transfected into NIH 3T3 fibroblasts (Fig. 1B). Thus, a consensus SMAD binding site seems necessary for TGF β -induction of CTGF. Previously, a sequence immediately downstream of this element was identified as being necessary for maximal TGF β induction of CTGF, and hence was called the TGF β RE (25). To compare the relative contribution of the SMAD site and the TGF β RE in terms of the TGF β induction of CTGF, we mutated the TGF β RE to a *Bam*HI site. In contrast to mutating the SMAD site, mutating the TGF β RE caused a marked decrease in basal CTGF expression, whereas still permitting a response to TGF β (Fig. 1C). Thus, the factor(s) binding to the TGF β RE are required for basal CTGF transcription and hence for the maximal response of the CTGF promoter to TGF β (25).

To determine whether SMAD proteins could bind to the putative SMAD binding site, we performed a gel shift assay with NIH 3T3 fibroblast nuclear extract, made from cells that had been treated with TGF β . A radiolabeled double-stranded oligonucleotide probe containing the putative SMAD binding site was used as probe (see Fig. 1 for sequence). We found that protein complexes could bind the probe (Fig. 2, arrow). This binding was specific as binding could be competed by adding excess unlabeled oligomer (Fig. 2). Binding of one of the complexes could be abolished by preincubating nuclear extract with an anti-SMAD3 or anti-SMAD4 antibody, but not with an anti-

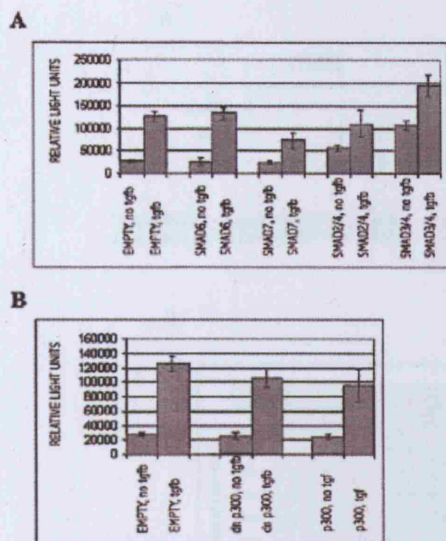


Fig. 3. Effect of transfecting SMAD expression vectors on CTGF promoter activity in NIH 3T3 fibroblasts. **A**, cotransfecting SMAD3 and SMAD4 potentially activates CTGF expression (SMAD 3/4). Cotransfecting SMAD2 and 4 were less effective at activating expression (SMAD 2/4). Transfecting SMAD7, but not SMAD6 blocked TGF β -induced gene expression. See Fig. 2 and "Materials and Methods" for details. **B**, cotransfecting a construct encoding wild-type p300 (p300) or dominant-negative p300 (dn p300) had no statistically significant effect on TGF β -induced CTGF expression. The full-length CTGF promoter/SEAP plasmid was used (0.5 μ g/well). An empty expression vector (EMPTT) was used as a control. Expression vectors were used at 1 μ g/well. Treatments had no effect on an SV40 promoter/enhancer/SEAP construct (CLONTECH) transfected in parallel (not shown). TGF β 2 was used at 25 ng/ml (tgfb). No tgf, no TGF β 2 added. Relative light units were based on SEAP expression (25 μ l of conditioned media) normalized to β -galactosidase activity (from a cotransfected CMV- β -galactosidase plasmid) are shown (mean \pm S.E., $n = 18$, except SMAD6 and 7, $n = 6$).

SMAD2 antibody (Fig. 2). Collectively, these data suggest that the putative SMAD site identified as being important for gene expression is a *bona fide* SMAD binding site. The components of the other complex are under further investigation.

To determine whether SMADs could regulate CTGF gene expression, we cotransfected expression vectors encoding various SMADs with a full-length CTGF promoter/SEAP construct into NIH 3T3 fibroblasts. Cotransfection of SMAD3 and SMAD4 enhanced CTGF expression significantly (Fig. 3A). However, cotransfection of SMAD2 and SMAD4 did not potentiate the TGF β -mediated induction of CTGF (Fig. 3A). Individually, only SMAD3 and SMAD4 mildly increased CTGF promoter activity in the absence of added TGF β , with SMAD4-inducing activity to approximately the same level as SMAD2 and SMAD4 combined (not shown). Transfection of the inhibitory SMAD, SMAD7, markedly attenuated the ability of TGF β and SMAD3 and 4 to increase CTGF promoter activity, but had little effect on basal expression (Fig. 3A). Conversely, transfecting the inhibitory SMAD, SMAD6, had no effect on the ability of TGF β to induce CTGF promoter activity (Fig. 3A). SMADs often require p300 as a transcriptional cofactor (32, 43). We found that transfecting either wild-type or dominant-negative p300 had no impact on the TGF β -induction of CTGF (Fig. 3B). Thus, SMADs modulate CTGF gene expression in a p300-independent fashion.

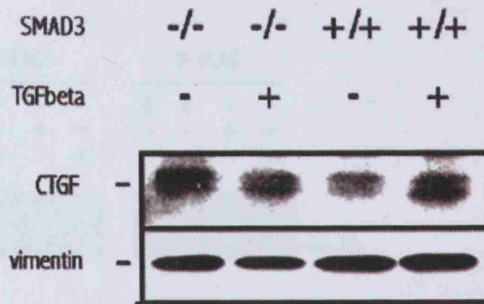


Fig. 4. TGF β does not induce CTGF in embryonic fibroblasts cultured from SMAD3-knockout mice. SMAD3-knockout and normal mouse embryonic fibroblasts (Ref. 45) were cultured and serum-starved for 24 h. Cells were then grown for an additional 24 h with and without addition of TGF β 2 (10 ng/ml). Western blots for CTGF were performed on the cell layer (25 μ g) as described under "Materials and Methods". TGF β induced CTGF in normal fibroblasts but not in SMAD3-knockout fibroblasts.

Our transfection data suggested that SMAD3 is principally involved in the TGF β -induction of CTGF. To verify this, we obtained fibroblasts cultured from SMAD3-knockout and wild-type fetal mice (45). We treated cells with TGF β for 24 h and assayed their ability to induce CTGF protein. Both wild-type and knockout embryonic mouse fibroblasts displayed constitutive CTGF expression. This result is not surprising, because CTGF protein is expressed constitutively in several cell types during development (for review, see Ref. 13). We note an elevated basal level of CTGF in the SMAD3-knockout cells, perhaps because of a compensation for a lack of inducibility by TGF β . However, although wild-type mouse fibroblasts responded to TGF β by elevating CTGF levels, fibroblasts cultured from SMAD3-knockout animals could not respond to TGF β (Fig. 4). Thus, the TGF β -mediated induction of CTGF requires SMAD3.

Regulators of SMADs Modulate TGF β -induced CTGF Gene Expression, ski and snoN.—To verify that SMADs activate CTGF gene expression, we then examined the ability of several known regulators of SMAD activity to modulate CTGF gene expression. Recently, the oncoproteins ski and snoN have been shown to suppress SMAD signaling in keratinocytes by binding directly to SMADs and thus preventing their participation in functional transcriptional complexes (46–48). We decided to use this fact to further probe the notion that TGF β -mediated induction of CTGF was SMAD-dependent and to determine whether this silencing mechanism was functional in fibroblasts.

To verify that ski and snoN could interact with SMADs in NIH 3T3 fibroblasts, we cotransfected expression vectors encoding FLAG-tagged SMADs 3 and 4 along with expression vectors encoding either HA-tagged ski or HA-tagged snoN into NIH 3T3 fibroblasts. The presence of these molecular tags allowed easy recognition and manipulation of the transfected proteins. After transfection, cells were serum-starved for 24 h and were then lysed. Because 20 min of TGF β treatment degrades snoN (47), we also wanted to ensure that transfected snoN existed even in the presence of exogenous TGF β . Thus, we also prepared lysates from cells that had been treated with TGF β for 20 min immediately after the serum-starvation treatment. To verify expression of transfected FLAG-tagged SMADs, whole cell lysate was then subjected to SDS-polyacrylamide gel electrophoresis. By Western blot analysis, transfected FLAG-tagged SMADs were readily detected with an

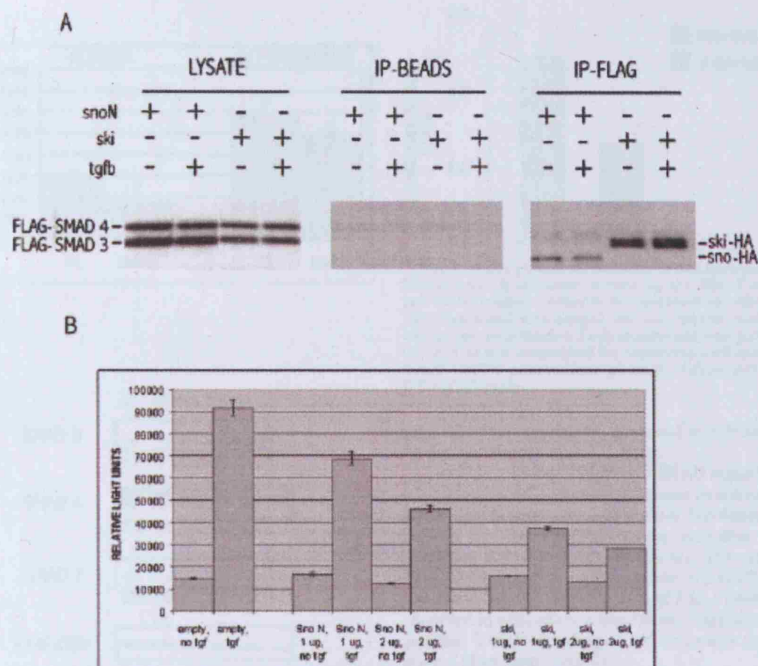


Fig. 5. ski and snoN block the TGF β induction of CTGF. *A*, ski and snoN bind to SMADs in NIH 3T3 fibroblasts. Cells were transfected with expression vectors encoding SMAD3 and SMAD4, which were both FLAG-tagged and either sno or ski, which were both HA-tagged (2 μ g of each expression vector per 100-mm dish). Cells were serum-starved for 24 h after transfection, after which cells were immediately lysed or treated for an additional 20 min with 25 ng/ml TGF β 2, as indicated (*tgfb*). Whole lysates (25 μ g/lane) were subjected to SDS-polyacrylamide gel electrophoresis and Western analysis with an anti-FLAG antibody, to verify expression of SMADs 3 and 4 (*LYSATE*). Equal amounts of lysate were then either immunoprecipitated with unconjugated agarose gel (*IP-BEADS*) or FLAG-agarose gel (*IP-FLAG*). Gels were then subjected to Western analysis with anti-HA antibody to detect transfected HA-tagged ski and snoN, which were detected only when anti-FLAG conjugated beads were used. *B*, ski and snoN block the TGF β - and SMAD-mediated induction of CTGF. Transfecting expression vectors encoding ski and snoN block the TGF β - and SMAD-mediated induction of CTGF. Transfecting different amounts of ski and snoN expression vectors (as shown) into NIH 3T3 fibroblasts block the ability of TGF β (25 ng/ml) to induce CTGF promoter activity. The full-length CTGF promoter/SEAP plasmid was used. Empty expression vector (*empty*) was used as a control at equal amounts to ski or snoN expression vector. Relative light units based on SEAP expression (25 μ l of conditioned media) normalized to β -galactosidase activity (from a cotransfected CMV- β -galactosidase plasmid) are shown (mean \pm S.E., $n = 6$).

anti-FLAG antibody in lysates from cells that has been cotransfected with either ski and snoN transfected cells (Fig. 5A, *LYSATE*).

To determine whether HA-tagged ski and snoN could bind to FLAG-tagged SMADs to inactivate them, we then immunoprecipitated lysates with an anti-FLAG antibody. In extracts from cells that had been cotransfected with HA-tagged ski and FLAG-tagged SMAD3 and SMAD4, we were able to immunoprecipitate HA-tagged ski with anti-FLAG-agarose gel, as seen with an anti-HA antibody (Fig. 5A, *IP-FLAG*). Conversely, no HA-tagged ski was immunoprecipitated when unconjugated beads were used (Fig. 5A, *IP-BEADS*). Similarly, in extracts from cells transfected with FLAG-tagged SMADs 3 and 4 and HA-tagged snoN, we were able to immunoprecipitate snoN with anti-FLAG-agarose gel (Fig. 5, *IP-FLAG*) but not with unconjugated agarose (Fig. 5A, *IP-BEADS*). We found that addition of TGF β to cells for 20 min before lysis had no detectable effect on anti-FLAG precipitable ski or snoN levels (Fig. 5A; TGF β -added lanes, *IP-FLAG*). Thus, transfected ski and snoN bound with SMAD3/4 in NIH 3T3 fibroblasts and thus could be used to test the idea that CTGF gene induction by TGF β is SMAD-dependent.

We then assessed the effect of transfected ski and snoN

constructs on CTGF promoter activity in NIH 3T3 fibroblasts when visualized by readout from a full-length CTGF promoter/SEAP reporter construct. We found that ski and snoN suppressed the induction of CTGF promoter activity by TGF β (Fig. 5B). There appeared to be a greater effect of ski than snoN on CTGF expression, presumably because TGF β treatment degrades snoN (47). Given the known abilities of ski and snoN (46–48), these results support the idea that SMADs modulate CTGF gene expression.

CTGF Expression in Scleroderma Fibroblasts Is Independent of SMAD Signaling—Previously, we showed that CTGF protein was constitutively expressed in fibroblasts cultured from lesional areas of scleroderma patients, even in the absence of exogenous TGF β (12, 20) and that this elevated expression was due at least in part to high levels of CTGF promoter activity (12). To investigate the role of SMAD-dependent signaling in the elevated level of CTGF characteristic of scleroderma, we transfected our full-length CTGF promoter/SEAP reporter construct into normal dermal or scleroderma fibroblasts and compared its expression level to that of our CTGF promoter/SEAP reporter construct with a mutated SMAD binding site. We used dermal fibroblasts cultured from four normal individuals and ten individuals with diffuse scleroderma. Mutation of the

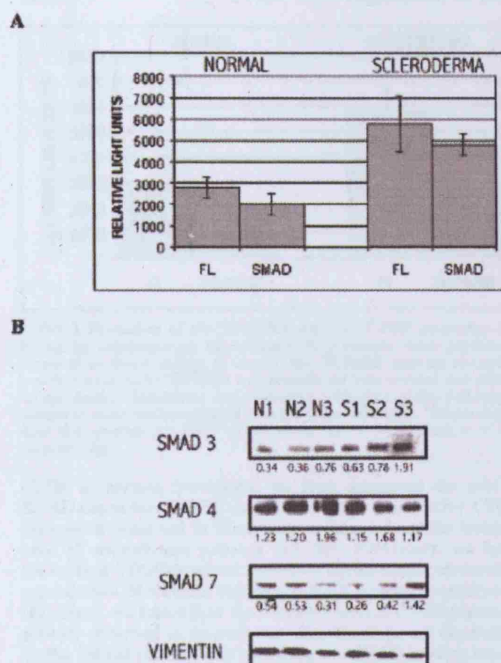


FIG. 6. Contribution of SMADs to basal CTGF expression in scleroderma fibroblasts. **A**, mutation of the SMAD binding element does not reduce the high level of CTGF promoter activity in scleroderma fibroblasts. Either the full-length CTGF promoter/reporter construct (FL) or the SMAD mutant promoter/reporter construct (SMAD) was transfected into normal dermal fibroblasts (from four individuals) or scleroderma fibroblasts (from ten individuals). Each experiment was performed in triplicate and normalized for transfection efficiency (with cotransfected CMV- β -galactosidase plasmid). Values represent the average (\pm S.E.) of all trials ($n = 12$ and $n = 30$, respectively). **B**, SMADs 3, 4, and 7 levels in normal and scleroderma fibroblasts. Densitometric analysis was performed as described under "Materials and Methods." Values shown are arbitrary densitometric units adjusted for vimentin levels in each lane. Fibroblasts cultured from three normal individuals (N1, N2, N3) and three individuals with scleroderma with the highest CTGF promoter activity (S1, S2, S3) are shown.

SMAD binding site had no statistically significant effect on the basal level of promoter activity either in normal or scleroderma fibroblasts (Fig. 6A; Student's unpaired t test, $p > 0.05$). Thus, the high level of CTGF promoter activity observed in scleroderma fibroblasts is not dependent on the SMAD recognition sequence.

To further clarify the potential role of SMADs in the scleroderma phenotype, we used Western blot analysis to compare the levels of expression of SMADs 3, 4, and 7 (because of their role in CTGF gene expression) in normal and scleroderma dermal fibroblasts. We used dermal fibroblasts cultured from three normal individuals and affected areas of three individuals with scleroderma. The scleroderma fibroblasts chosen had the highest levels of CTGF promoter activity. Bands were quantified relative to a vimentin standard (Fig. 6B). Although some individuals showed elevated levels of particular SMAD proteins, no overall picture emerged which correlated SMAD levels with the scleroderma phenotype; for example individual S3 had the highest SMAD3 level but also had extremely high SMAD7 (Fig. 6B). That is, our results suggest that the elevated

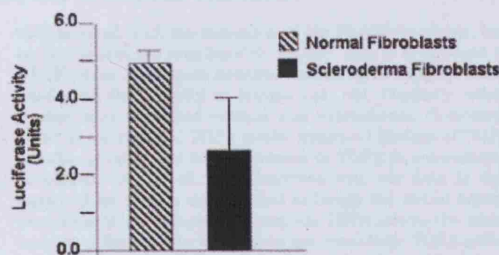


FIG. 7. PAL-1 promoter activity is not elevated in scleroderma fibroblasts. A construct containing the SMAD-responsive 800-base pair PAL-1 promoter linker to the luciferase reporter gene (Refs. 49, 50) was transfected into normal (two individuals) and scleroderma fibroblasts (two individuals). Each experiment was performed in triplicate ($n = 6$) and normalized for transfection efficiency using a cotransfected CMV- β -galactosidase plasmid. Values reflect the average (\pm S.E.) of all trials.

level of CTGF expression observed in scleroderma appears to be independent of SMAD action.

To further clarify if elevated SMAD signaling was responsible for activated expression of genes in scleroderma, we transfected into scleroderma and normal fibroblasts a construct containing the human plasminogen activator inhibitor (PAL-1) promoter linked to firefly luciferase. This construct has been previously shown to be responsive to SMAD-dependent TGF β signaling (49, 50). No elevation of PAL-1 promoter activity was observed in scleroderma fibroblasts, suggesting that SMAD-dependent TGF β signaling is not generally activated in scleroderma fibroblasts (Fig. 7).

To determine whether the elevated level of CTGF promoter activity in scleroderma cells reflected elevated level of basal promoter activity, we transfected the TGF β RE-mutant CTGF promoter/SEAP reporter construct into normal and scleroderma fibroblasts. We found that mutating the TGF β RE reduced CTGF promoter levels in both normal and scleroderma cells (Fig. 8). In fact, removing this element reduced CTGF promoter levels observed in scleroderma fibroblasts to that of levels in normal fibroblasts, suggesting that this element is involved with the high level of CTGF expression observed in scleroderma.

DISCUSSION

TGF β is known to activate gene expression through the action of SMAD proteins (25, 26). In the absence of TGF β , SMADs 2 and 3 are primarily cytosolic. When TGF β is present, SMADs 2 and 3 are phosphorylated by the TGF β receptor, bind to SMAD 4, and migrate into the nucleus to activate expression of TGF β -responsive genes. The inhibitory SMADs, SMAD6 and SMAD7, antagonize this pathway of signaling by TGF β family members (39, 40). For the CTGF promoter, we found that transfecting SMADs 3 and 4 into fibroblasts enhanced CTGF promoter activity whereas SMAD7, but not SMAD6, suppressed TGF β -induced CTGF expression. In SMAD3-knockout fibroblasts (45), CTGF induction did not occur indicating that SMAD2 could not substitute for SMAD3. We also found that ski and snoN oncoproteins, which bind SMADs thereby blocking SMAD-dependent gene expression (46–48), attenuated the TGF β -mediated induction of CTGF. That SMADs are involved with induction of CTGF is not surprising because SMADs have been specifically implicated in the TGF β up-regulation of matrix genes (37, 51).

In adults, constitutive CTGF expression is a consistent marker of the fibrotic phenotype (12, 20–24). After we established the role of SMADs in the TGF β -mediated induction of

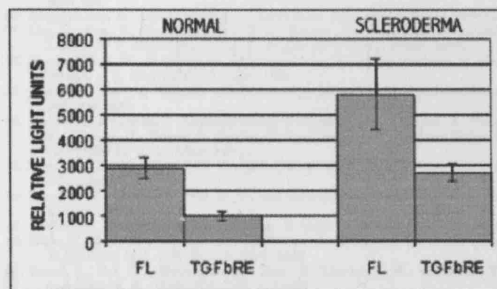


Fig. 8. Mutation of the TGF β RE reduces CTGF promoter activity in scleroderma fibroblasts. Experiments were performed identical to those in Fig. 6, except the TGF β RE mutant promoter/reporter construct (TGF β RE) was transfected into normal and scleroderma dermal fibroblasts and compared with that of the full-length promoter construct transfected into the same cells (FL). Values represent the average (\pm S.E.) of all trials (i.e. $n = 12$ and $n = 30$, respectively).

CTGF in normal fibroblasts, we then examined the role of SMAD-dependent TGF β signaling on the constitutive CTGF expression observed in fibroblasts cultured from the lesional area of scleroderma patients (12, 20). Previously, we have shown that CTGF promoter activity is substantially elevated in scleroderma fibroblasts relative to their normal counterparts (12). Here, we found that the elevated level of CTGF promoter activity observed in scleroderma fibroblasts is not dependent on the SMAD binding site; mutating the SMAD binding site in the context of the CTGF promoter does not decrease CTGF promoter activity in scleroderma fibroblasts. Conversely the previously termed TGF β RE (25) is required for basal CTGF promoter activity in normal fibroblasts and also for the elevated activity in scleroderma fibroblasts. The factor(s) binding this element are currently under investigation, but are not AP-1, CREB, or Sp1; factors known to contribute to TGF β responses in other contexts, because oligonucleotides containing consensus binding sites for these factors do not compete for protein binding to the TGF β RE in gel shift studies.² In any event, the difference in gene expression patterns between normal and scleroderma fibroblasts is not solely caused by activated SMAD-dependent TGF β signaling because the SMAD-responsive PAI promoter (49, 50) is not elevated in scleroderma fibroblasts.

Collectively, these results suggest that the maintenance of the scleroderma phenotype, as visualized by elevated CTGF levels, is not caused by SMAD-dependent TGF β signaling. These data do not exclude the possibility that a cryptic SMAD site in the CTGF promoter is used in the scleroderma fibroblasts; however, sequence inspection of the CTGF promoter does not yield another SMAD consensus binding site. In addition, cotransfection of SMAD3 and 4 into dermal fibroblasts does not activate expression of our SMAD mutant CTGF promoter/reporter construct (not shown). Furthermore, TNF α treatment, which suppresses the TGF β -induction of collagen and CTGF in a manner possibly involving elevation of SMAD7 (20, 53–55), has no effect on basal collagen or CTGF gene expression in scleroderma fibroblasts (20).

These results are consistent with previous studies that have examined the role of TGF β in acquisition and maintenance of the scleroderma phenotype. For example, recent reports localized TGF β mRNA to the leading edge of the scleroderma lesion, that is the region of enhanced inflammatory response presum-

ably involved with the initiation of the fibrotic response, but not in the lesional area itself (9, 56, 57). This is in contrast to CTGF whose expression patterns and levels are highly correlated with the severity of fibrosis (44, 58). Similarly, other studies that examined normal and scleroderma fibroblasts failed to see elevated TGF β levels, enhanced binding of TGF β to cells, or enhanced responsiveness to TGF β in scleroderma fibroblasts (10, 11, 12, 52). Combined with our data in this report, these results suggest that although the initial expansion of the sclerotic lesion may require TGF β action, the maintenance of the sclerotic lesion does not result from TGF β action *per se*. Rather, the maintenance of the scleroderma phenotype may result from the failure to suppress downstream responses to TGF β , such as the induction of CTGF and collagen synthesis. Thus although anti-TGF β /anti-SMAD strategies may be effective in blocking the onset or progression of fibrosis (see Ref. 6), these strategies may not be effective at reversing fibrosis once it has occurred. Given the known profibrotic activity of CTGF, the high level of constitutive, SMAD-independent, CTGF expression present in the sclerotic lesion may be a cause of the excessive scarring that is observed in scleroderma patients. Thus, an anti-CTGF strategy, or at least identifying why CTGF is overexpressed in fibrosis, might yield a more effective method of reversing the fibrotic process.

Acknowledgments—We thank George Martin for critical review of the manuscript, Alain Mauviel and John Varga for technical advice, Joan Massague for SMADs 2, 3, 4, 6, and 7 expression vectors, Anita Roberts for SMAD3-knockout fibroblasts, Robert Weinberg for ski and smnN expression vectors, and Dan Rifkin for the PAI-1/luciferase construct.

REFERENCES

- Gruschwitz, M., Muller, P. U., Sepp, N., Hofer, E., Fontana, A., and Wick, G. (1990) *J. Invest. Dermatol.* 94, 197–203.
- Hayashida, A., Koch, J., Schuppan, D., Modder, W. C., and Hahn, E. G. (1991) *J. Hepatol.* 13, 328–333.
- Peltonen, J., Kahari, L., Uitto, J., and Jimenez, S. A. (1990) *Arthritis Rheum.* 33, 1389–1395.
- Black, C. M., and Denton, C. P. (1990) *Oxford Textbook of Rheumatology*, pp. 771–789. Oxford University Press, New York.
- McCartney-Francis, N. L., Frazier-Jessen, M., and Wahl, S. M. (1998) *Int. Rev. Immunol.* 16, 553–590.
- Blöbe, G. C., Schiemann, W. P., and Lodish, H. F. (2000) *N. Engl. J. Med.* 342, 1350–1358.
- Smith, E., and Leroy, E. C. (1990) *J. Invest. Dermatol.* 95, 125a–127a.
- Deguchi, Y. (1992) *Ann. Rheum. Dis.* 51, 362–365.
- Querfeld, C., Eckes, B., Hueckamp, C., Kreig, T., and Sollberg, S. J. (1999) *J. Dermatol. Sci.* 21, 18–22.
- Moreland, L. W., Goldsmith, K. T., Russell, W. J., Young, K. R., Jr., and Garver, R. L., Jr. (1992) *Ann. J. Med.* 93, 628–636.
- Needleman, B. W., Choi, J., Burrows-Meza, A., and Fontana, J. A. (1990) *Arthritis Rheum.* 33, 650–656.
- Shi-wen, X., Pennington, D., Holmes, A., Leask, A., Bradham, D., Beauchamp, J. R., Fonseca, C., du Bois, R. M., Martin, G. R., Black, C. M., and Abraham, D. J. (2000) *Exp. Cell Res.* 259, 213–224.
- Mousad, E. E. A., and Brigstock, D. A. (2000) *Mol. Genet. Metab.* 71, 276–282.
- Bradham, D. M., Igarashi, A., Potter, R. L., and Grotendorst, G. (1991) *J. Cell Biol.* 114, 1285–1294.
- Brigstock, D. R., Steffen, C. L., Kim, G. Y., Vegunta, R. K., Diehl, J. R., and Harding, P. A. (1997) *J. Biol. Chem.* 272, 20275–20282.
- Grotendorst, G. R. (1997) *Cytokine Growth Factor Rev.* 8, 171–179.
- Duncan, M. R., Frazier, K. S., Abramson, S., Williams, S., Klapper, H., Huang, X., and Grotendorst, G. R. (1999) *FASEB J.* 13, 1774–1786.
- Shimo, T., Nakanishi, T., Kimura, Y., Nishida, T., Ishizaki, K., Matsumura, and Takigawa, M. (1998) *J. Biochem. (Tokyo)* 124, 130–140.
- Mori, T., Kawara, M., Shiozaki, N., Hayashi, T., Kakimura, A., Igarashi, M., Takigawa, T., Nakanishi, T., and Takehara, K. (1999) *J. Cell. Physiol.* 181, 153–159.
- Abraham, D. J., Shiven, X., Black, C. M., Sa, S., Xu, Y., and Leask, A. (2000) *J. Biol. Chem.* 275, 15225–15235.
- Dammeyer, J., Brauchle, M., Falk, W., Grotendorst, G. R., and Werner, S. (1998) *Int. J. Biochem. Cell Biol.* 30, 909–922.
- di Mola, F. F., Friess, H., Martignoni, M. E., Di Sebastiano P., Zimmermann, A., Innocenti, P., Graber, H., Gold, L. I., Kore, M., and Buchler, M. W. (1999) *Ann. Surg.* 230, 63–71.
- Igarashi, A., Nishiro, K., Kikuchi, K., Sato, S., Iha, H., Fujimoto, M., Grotendorst, G. R., and Takehara, K. (1996) *J. Invest. Dermatol.* 106, 729–733.
- Ito, Y., Aten, J., Bende, R. K. J., Oemar, B. S., Rabelink, T. J., Weening, J. J., and Goldschmeding, R. (1998) *Kidney Int.* 53, 853–861.
- Grotendorst, G. R., Okochi, H., and Hayashi, N. (1996) *Cell Growth Differ.* 7,

²A. Leask and S. Sa, unpublished data.

- 469-480
26. Kretschmer, M., and Massagué, J. (1998) *Curr. Opin. Genet. Dev.* 8, 108-111
27. Liu, X., Sun, Y., Constantinescu, S. N., Karam, E., Weinberg, R. A., and Lodish, H. F. (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94, 10666-10674
28. Macias-Silva, M., Abdollah, S., Hoodless, P. A., Pirone, R., Attianese, L., and Wrangé, J. L. (1998) *Cell* 97, 1215-1224
29. Legoux, G., Hata, A., Hatzidimitrakou, A., and Massagué, J. (1998) *Nature* 393, 822-826
30. Nakao, A., Inamura, T., Souchelnytskyi, S., Kurokawa, M., Ishizaki, A., Oeda, E., Tanaka, K., Hattori, J., Hattori, C. H., Miyazono, K., and ten Dijke, P. (1997) *EMBO J.* 16, 5352-5362
31. Hua, X., Liu, X., Ansari, D. O., and Lodish, H. F. (1998) *Genes Dev.* 12, 3084-3095
32. Feng, X. H., Zhang, Y., Wu, R. Y., and Derynck, R. (1998) *Genes Dev.* 12, 2155-2163
33. Jauch, R., Wells, N. J., and Hunter, T. (1998) *Genes Dev.* 12, 2114-2119
34. Shen, X., Hu, P. P., Liberati, N. T., Datto, M. B., Frederick, J. P., and Wang, X. F. (1998) *Mol. Cell. Biol.* 18, 3309-3319
35. Zou, L., Dai, J. L., Buchbinder, P., Zhou, S., Kinzler, K. W., Vogelstein, B., and Kraus, S. E. (1998) *Mol. Cell* 1, 11-17
36. Zhang, Y., Feng, X. H., and Derynck, R. (1998) *Nature* 394, 900-915
37. Vindemogel, L., Kon, A., Lechman, R. J., Uitto, J., Roberts, A. B., and Mauviel, A. (1998) *J. Biol. Chem.* 273, 18065-18067
38. Jonk, L. J., Itoh, S., Haddad, C. H., ten Dijke, P., and Krüger, W. (1998) *J. Biol. Chem.* 273, 21145-21152
39. Adhikari, M., Mores, A., Jomaa, S., Itoh, S., Sampath, K., Westmark, B., Haddad, C. H., Haddad, N. E., and ten Dijke, P. (1998) *Biochem. Biophys. Res. Commun.* 248, 505-511
40. Roberts, A. B. (1999) *Microbes Infect.* 1, 1265-1273
41. LeRoy, E. C. (1974) *J. Clin. Invest.* 54, 880-886
42. Leach, A., Byrne, C., and Fuchs, E. (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88, 7048-7052
43. Jauch, R., Wells, N. J., and Hunter, T. (1998) *Genes Dev.* 12, 2114-2119
44. Sato, S., Nagao, T., Hasegawa, M., Tamatani, T., Nakazaki, T., Takigawa, M., and Takahara, K. (2000) *J. Rheumatol.* 27, 149-154
45. Ashcroft, G. S., Yang, X., Glick, A. B., Weinstein, M., Lettino, J. L., Mizel, D. E., Anzano, M., Grosswell-Wild, T., Wahl, S. M., Dang, C., and Roberts, A. B. (1999) *Nat. Cell Biol.* 1, 260-266
46. Luo, K., Storchman, S. L., Wang, W., Chen, D., Martens, E., Zhou, S., and Zhou, Q. (1999) *Genes Dev.* 13, 2194-2200
47. Storchman, S. L., Wang, W., Zhou, S., Zhou, Q., and Luo, K. (1999) *Science* 286, 771-774
48. Sun, Y., Liu, X., Ng-Eaton, E., Lodish, H. F., and Weinberg, R. A. (1999) *Proc. Natl. Acad. Sci.* 96, 12443-12447
49. Abo, M., Hargal, C. N., Metz, C. N., Nunes, I., Lockstott, N., and Rifkin, D. B. (1994) *Anal. Biochem.* 216, 274-284
50. Yamamura, Y., Hua, X., Bergelson, S., and Lodish, H. (2000) *J. Biol. Chem.* 275, 36295-36302
51. Chen, S. J., Yuan, W., Mori, Y., Lovinson, A., Trujanowicz, M., and Varga, J. (1999) *J. Invest. Dermatol.* 113, 49-57
52. McWhirter, A., Colosetti, P., Rubin, K., Miyazono, K., and Black, C. (1994) *Lab. Invest.* 71, 885-895
53. Inagaki, Y., Truter, D., Tanaka, S., DiLiberato, M., and Ramirez, F. (1996) *J. Biol. Chem.* 271, 3353-3359
54. Verrecchia, F., Passah, M., Atti, A., and Mauviel, A. (2000) *J. Biol. Chem.* 275, 30236-30241
55. Bitens, M., von Gersdorff, G., Liang, D., Dominguez-Rodriguez, A., Berg, A. A., Rajkumar, M., and Bottinger, E. P. (2000) *Genes Dev.* 14, 187-197
56. Nakao, A., Fujii, M., Matsunaga, R., Kamano, K., Saito, Y., Miyazono, K., and Iwamoto, I. (1999) *J. Clin. Invest.* 104, 5-11
57. Higley, H., Paruchuri, K., Chen, S., Wang, W., Vanchaswaran, R., and Black, C. (1994) *Arthritis Rheum.* 37, 276-283
58. Iguchi, A., Nishio, K., Kikuchi, K., Sato, S., Ito, I., Gotschardt, G. R., and Takahara, K. (1998) *J. Invest. Dermatol.* 110, 290-294

Constitutive Connective Tissue Growth Factor Expression in Scleroderma Fibroblasts Is Dependent on Sp1*

Received for publication, May 13, 2003, revised July 11, 2003
Published, JBC Papers in Press, July 29, 2003, DOI 10.1074/jbc.M306019200

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Fibrotic diseases such as scleroderma (systemic sclerosis, SSc) are characterized by an excessive production of extracellular matrix and profibrotic proteins such as connective tissue growth factor (CTGF). In normal dermal fibroblasts, CTGF is not expressed unless induced by proteins such as tumor growth factor- β (TGF β). Conversely, in fibroblasts cultured from fibrotic lesions CTGF mRNA and protein are constitutively expressed, even in the absence of exogenously added TGF β . Thus, studying the mechanism underlying CTGF overexpression in SSc fibroblasts is likely to yield valuable insights into the basis of the fibrotic phenotype of SSc and possibly other scarring disease. CTGF overexpression is mediated primarily by sequences in the CTGF promoter. In this report, we identify the minimal promoter element involved with the overexpression of CTGF in SSc fibroblasts. This element is distinct from the element necessary and sufficient for the induction of CTGF expression by TGF β in normal fibroblasts. Within this region is a functional Sp1 binding site. Blocking Sp1 activity reduces the elevated, constitutive levels of CTGF promoter activity and protein expression observed in SSc fibroblasts. Relative to those prepared from normal dermal fibroblasts, nuclear extracts prepared from SSc fibroblasts possess increased Sp1 binding activity. Removal of phosphate groups from nuclear extracts enhanced Sp1 binding activity, suggesting that phosphorylation of Sp1 normally reduces Sp1 binding to DNA. Thus, the constitutive overexpression of CTGF in SSc fibroblasts seems to be independent of TGF β signaling but dependent at least in part on Sp1.

has long been devoted to the potential role of this factor in initiation and maintenance of fibrosis (for reviews, see Refs. 3 and 4). For example, there is a clear correlation between TGF β action and the initiation of fibrosis; in acute drug- or surgery-induced animal models, anti-TGF β strategies, such as a neutralizing TGF β antibody or overexpression of Smad7 (for reviews, see Refs. 3 and 4), are effective at attenuating the onset and severity of fibrogenesis.

However, the precise role that TGF β plays in human fibrotic disease is not entirely clear. In the chronic fibrosing disorder scleroderma (SSc) TGF β mRNA is localized to the leading edge of the fibrotic lesion (i.e. to the region of enhanced inflammatory response that is presumably involved with the expansion of the fibrotic response) but not to the lesion area itself (5). Although a general feature of fibroblasts taken from SSc lesions is that they show elevated levels of collagen relative to their normal counterparts, SSc fibroblasts do not show elevated TGF β levels or binding activity or enhanced sensitivity to TGF β treatment (6–8). Thus, TGF β expression seems to be clearly associated with the initiation of the fibrotic phenotype of SSc; however, the role of this cytokine in the maintenance of the fibrotic phenotype in SSc, or of other fibrotic diseases, for that matter, remains unclear.

In contrast to TGF β , expression of the profibrotic protein CTGF correlates well with the severity of fibrotic phenotype of SSc (9, 10). Although not normally expressed in fibroblasts unless induced by TGF β or other proteins, such as thrombin, CTGF is constitutively expressed in many fibrotic disorders, such as scleroderma, liver sclerosis, and pulmonary and renal fibrosis (for reviews, see Refs. 11 and 12). CTGF protein induces proliferation, collagen synthesis, and chemotaxis in mesenchymal cells (13–15). CTGF seems to be associated with matrix deposition. For example, a CTGF response element lies between nucleotides –376 and +58 of the type I collagen (Col1A2) promoter (16), which includes the TGF β response element of this promoter (17). In addition, whereas subcutaneous injection of TGF β into neonatal rats results only in a transient fibrotic response, coinjection of CTGF and TGF β results in sustained fibrosis (18). This result could possibly be because CTGF can bind TGF β and consequently may enhance the activity of TGF β , at low concentrations, to bind its receptors (19). Collectively, these results suggest that TGF β may initiate, but CTGF may sustain, the fibrotic response. Thus, examining the mechanism underlying the constitutive overexpression of CTGF in fibroblasts from fibrotic lesions should provide insights into the molecular mechanism underlying fibrosis.

CTGF expression seems to be controlled primarily at the level of transcription and involves both TGF β -dependent and -independent mechanisms (20–24). For example, the TGF β

Wound healing requires the *de novo* synthesis of connective tissue. If this process is not appropriately terminated, excessive matrix deposition occurs, resulting in pathological fibrosis (1, 2). Fibrotic diseases are among the largest groups of disease for which there is no known effective therapy, in part because the cause of these diseases remains elusive. Because TGF β ¹ promotes fibroblast proliferation and matrix synthesis, attention

* This work was supported in part by National Institutes of Health Grant AR45879, The Arthritis Research Campaign (United Kingdom), The Raynaud's and Scleroderma Association, the Welton Foundation, and the Nightingale Charitable Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: TGF β , tumor growth factor- β ; SSc, scleroderma; CTGF, connective tissue growth factor; CMV, cytomegalovirus; SEAP, secreted alkaline phosphatase; wt, wild type.

induction of the CTGF promoter requires consensus binding motifs for Smad and TEF transcription factors (22–24). Similarly, the elevated expression of CTGF protein observed in SSc fibroblasts is paralleled by elevated levels of CTGF promoter activity (20, 22). Thus, analyzing the relative contribution of TGF β -dependent and -independent mechanisms to the elevated level of CTGF promoter activity in lesional SSc fibroblasts should yield valuable insights into the molecular basis of the maintenance of the SSc phenotype.

To gain insights into the molecular mechanism underlying the fibrotic phenotype of SSc, in this study, we identify regions of the CTGF promoter necessary for the overexpression of CTGF in lesional, dermal SSc fibroblasts. Our results provide new insights into the molecular mechanism underlying the maintenance of the fibrotic phenotype of the SSc fibroblast.

MATERIALS AND METHODS

Cell Culture, Reporter Assays, Transfections, and Western Analysis.—Dermal fibroblasts from SSc lesions and healthy persons were taken from biopsies of age-, sex-, and anatomical site-matched volunteers, after informed consent was obtained. All patients fulfilled the criteria of the American College of Rheumatology for the diagnosis of diffuse SSc. Fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and used between passages 2 and 5 (18, 20). Transfections and reporter assays were carried out as described previously (20, 22), using 1 μ g of a reporter construct and 0.25 μ g of the control CMV β -galactosidase plasmid (Clontech). For assays in which the role of trans-acting proteins were to be tested, 0.5 μ g of reporter and 1 μ g of empty vector or expression vector encoding the protein of interest were used. TGF β 2 was from R&D Systems or Celtrix. Data presented are means \pm S.E. Statistical analysis was performed by the Student's unpaired test, *p* values less than 0.05 were considered statistically significant. For Western blots, cells were cultured and 25 μ l of media were electrophoresed through a 12% SDS/polyacrylamide gel (Novex) and blotted to nitrocellulose (Bio-Rad). CTGF protein was detected as described previously (20, 22). For studies involving mithramycin, 150 nM mithramycin (Sigma) was added 6 h before harvesting. Anti-actin antibody was from Sigma.

DNA Constructs.—The CTGF promoter/SEAP reporter constructs were as described previously (20, 22). Point mutations in putative Sp1 sites were introduced (Stratagene) using primer (Sigma Genosys) for the 5' Sp1 mutant 5'-CAGGTCAATGCGGAGCGCGATATCCCG-GAGCGTATAAAGCCTC-3' and for the 3' Sp1 mutant 5'-GTATA-AAAGCCTCGGCGCGCGCGCCCAACTCACACAAC-3'. The Sp1 expression vector was provided by Dimitris Kardassis (University of Crete, Greece).

Electrophoretic Mobility Shift Assays.—An enzyme-linked immunosorbent assay detecting levels of Sp1 binding to a double-stranded oligomer containing a consensus Sp1 site was purchased (Clontech). A commercially available source of Sp1 was purchased (Promega). Fibroblast nuclear extracts were prepared and quantified as described previously (25). Gel shifts were performed with 2–5 μ g of protein and a 0.5-ng probe (1.5×10^4 cpm) as described previously (22). For competition experiments, 100-fold excess unlabeled competitor was added to the reaction mixture before incubation, when required. The SMAD oligomer was described previously (25); AP2 and Sp1 oligomers were purchased (Promega). A gel-shift microarray that contained double-stranded oligomers bearing consensus binding sites for known transcription factors was purchased and used as described by the manufacturer (Panomics). To assess the role of phosphorylation in Sp1 binding, nuclear extracts were incubated with and without 1 unit of calf intestinal alkaline phosphatase (Roche) for 1 h at 37 °C before use in an electrophoretic mobility shift assay.

RESULTS

The Region Lying between Nucleotides -86 and +17 of the CTGF Promoter Is Required for Its Overexpression in SSc Fibroblasts.—To identify regions of the CTGF promoter important for its overexpression in SSc fibroblasts, we transfected a series of CTGF promoter deletion constructs (wt, -244 bp, and -86 bp) into normal and SSc dermal fibroblasts. All constructs contained abundant CTGF promoter expression, suggesting that the elevated level of CTGF expression observed in SSc fibroblasts was controlled by the first 86 base pairs upstream of

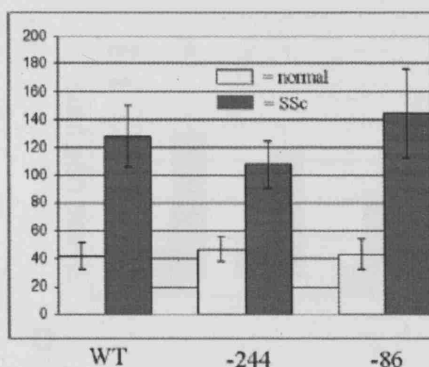


Fig. 1. Deletion analysis of the CTGF promoter: sequences required for its overexpression in SSc fibroblasts. CTGF promoter/SEAP reporter constructs (20) were transfected into dermal fibroblasts cultured from four healthy subjects as well as lesional areas from four subjects with diffuse SSc (*n* = 12). Constructs were: the wild-type CTGF promoter (WT, containing nucleotides -805 to +17), -244 (containing nucleotides -244 to +17), and -86 (containing nucleotides -86 to +17). Expression values (mean \pm S.D.), after adjusting for differences in transfection efficiencies among samples using a cotransfected vector (CMV β -galactosidase (Clontech)), are shown.

the transcription initiation start site (Fig. 1). This region of the CTGF promoter does not contain the TGF β response that is necessary and sufficient for TGF β to induce CTGF in fibroblasts (22–24).

The Region between -86 and +17 of the CTGF Promoter Contains One Functional Sp1 Site.—To begin to identify elements of the CTGF promoter lying between -86 and +17 that might contribute to the elevated level of CTGF expression in SSc fibroblasts, we examined this region of the CTGF promoter for consensus sites of known transcription factors. We noted a TATA box flanked by two consensus binding sites for transcription factor Sp1, one 5' to the TATA box (5' Sp1; Fig. 2A); the other 3' to the TATA box (3' Sp1; Fig. 2A). To determine whether the CTGF promoter's putative Sp1 elements were functional, we first assessed whether these sites could bind Sp1 protein. To do this, we synthesized and radiolabeled a double-stranded oligomer containing both putative Sp1 sites (the sequence shown in Fig. 2A) and performed gel-shift analyses using a commercially available source of Sp1 protein. We found that addition of Sp1 protein to the probe resulted in a shift that could be competed either by a 100-fold molar excess of either unlabeled probe or by an oligomer containing a consensus Sp1 site (Fig. 2B). However, a 100-fold molar excess of oligomers containing either a consensus AP2 site or the SMAD element of the CTGF promoter (22) did not compete for factor binding.

To test which of the putative Sp1 binding sites in the CTGF promoter could bind Sp1, we generated double-stranded oligomers that contained mutations in either the 3' Sp1 site or the 5' Sp1 site but were otherwise identical to the gel-shift probe and used them as competitors in our gel-shift assays. We found that a 100-fold molar excess of an oligomer containing a mutated 3' Sp1 site did not compete for factor binding; however, an oligomer containing a mutation in the 5' Sp1 site still competed for factor binding (Fig. 2B). Thus, Sp1 or Sp1-like binding activity binds to the consensus Sp1 site downstream from the TATA box in the CTGF promoter but not to the consensus site upstream of the TATA box.

To verify that the Sp1 binding site downstream of the TATA box was functional, we mutated the 5' Sp1 or the 3' Sp1 sites in the context of an otherwise wild-type, full-length CTGF pro-

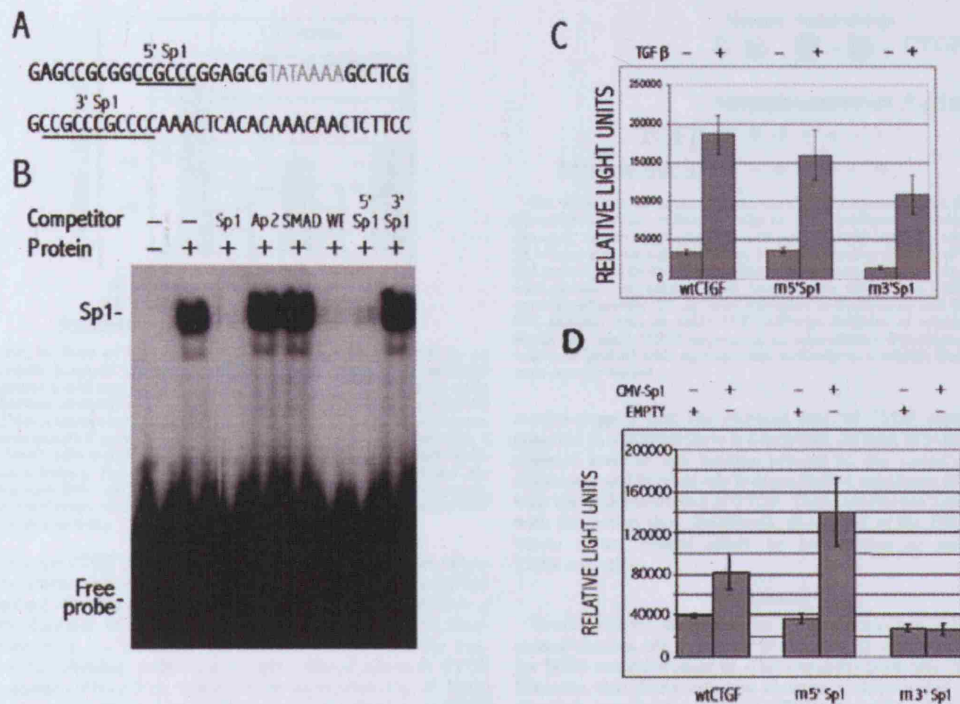


FIG. 2. Sp1 activates the CTGF promoter. **A**, sequence of the CTGF promoter. Putative Sp1 sites are underlined (5'Sp1 and 3'Sp1) and are defined relative to the TATA box (gray). **B**, the sequence shown in **A** was synthesized as a double-stranded oligomer, radiolabeled, and used in a gel-shift assay with HeLa nuclear extracts that contained Sp1 binding activity (Promega). Competitor oligonucleotides were used as indicated at 100-fold molar excess. The 5'Sp1 and 3'Sp1 competitors are identical to the probe (WT), other than that they contain a mutation to a HindIII (AAGCTT) site in the appropriate putative binding site. Oligomers bearing consensus binding sites for known transcription factor binding sites (Ap2 and Sp1) were from Promega. Mutation of the 3'Sp1 site abolishes the ability of the competitor oligomer to compete for Sp1 binding to the labeled probe. **C**, effect of mutating putative Sp1 sites on basal and TGFβ-induced CTGF promoter activity. The wild-type CTGF promoter/SEAP reporter construct (wtCTGF, containing nucleotides -806 to +17 (20)) or otherwise identical constructs bearing mutations in either 5'Sp1 or 3'Sp1 site of the CTGF promoter (see "Materials and Methods") were transfected into NIH 3T3 cells. After an 18-h incubation in serum-free media, TGFβ2 (25 ng/ml) was added for a further 24 h. Mutating the Sp1 site 3' to the TATA box (m3'Sp1) results in an equivalent reduction of basal promoter activity and TGFβ induction of CTGF; that is, mutation of the functional Sp1 site has no impact on the fold-induction of the promoter by TGFβ. All experiments were performed in 6-well plates. Cells were cotransfected with a CMV promoter-driven vector encoding β-galactosidase (0.25 μg) to control for differences in transfection efficiency. Values expressed are mean ± S.E. (n = 6). **D**, Sp1 transactivates the CTGF promoter. A wild-type CTGF promoter/SEAP reporter construct (wtCTGF (20)) or otherwise identical constructs bearing mutations in the Sp1 sites within the CTGF promoter at positions 5' (m5'Sp1) or 3' (m3'Sp1) of the TATA box (see Fig. 1) were transfected into NIH 3T3 cells. Cells were cotransfected with empty expression vector or vector encoding Sp1, as shown. All experiments were performed in 6-well plates. Cells were cotransfected with a CMV promoter-driven vector encoding β-galactosidase (0.25 μg) to control for differences in transfection efficiency. Values expressed are mean ± S.D. (n = 6).

motor, and subcloned the resultant fragments in front of the SEAP reporter gene. These constructs (m5'Sp1 and m3'Sp1, respectively; Fig. 2C) were transfected into fibroblasts. When we compared the expression levels of these new constructs with that of the wild-type, full-length CTGF promoter/SEAP reporter construct (wt CTGF; Fig. 2C), we found that mutation of the 3'Sp1 site, but not the 5'Sp1 site resulted in a decrease of ~50% in basal promoter activity. Thus, only the putative Sp1 site downstream from the TATA box contributed to the basal CTGF promoter activity. We then cotransfected NIH 3T3 fibroblasts with either empty expression vector or expression vector encoding Sp1 along with the wt CTGF, m5', and m3' promoter/reporter constructs. We found that Sp1 activated the wild-type reporter and the m5'Sp1 mutant construct, but not the m3'Sp1 mutant construct (Fig. 2D). Collectively, these data suggest that the Sp1 recognition motif 3' to the TATA box, but not the Sp1 site 5' to the TATA box, is functional and contributes

significantly to basal activity of the CTGF promoter.

To verify and extend previous results showing that this region of the CTGF promoter did not contain its TGFβ response element and to verify that Sp1 binding was not required for this response (22–24), we assessed whether the 3' Sp1 site was necessary for the TGFβ-mediated-induction of the CTGF promoter. To do this, we transfected the wild-type (wt CTGF), m3'Sp1, or m5'Sp1 mutant constructs into cell lines (Fig. 2C). After an 18-h incubation in serum-free media, cells were incubated for 24 h with or without 25 ng/ml TGFβ2. TGFβ significantly activated all the CTGF promoter/reporter constructs, indicating that Sp1 is not required for the TGFβ induction of the CTGF promoter.

Sp1 Is Required for the Elevated Levels of CTGF Expression Observed in Scleroderma Fibroblasts.—To assess the contribution of Sp1 to the elevated level of CTGF expression in SSc fibroblasts, we transfected these cells with the full-length,

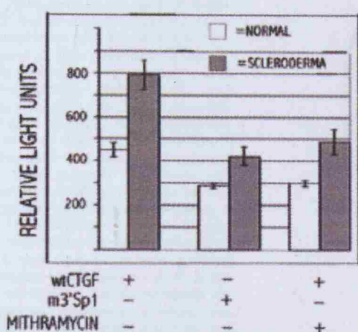


Fig. 3. Role of Sp1 in elevated CTGF promoter activity in dermal, lesional scleroderma fibroblasts. CTGF promoter/SEAP reporter constructs used contained either the wtCTGF (Fig. 3) or an otherwise identical construct containing a mutated SMAD site (Δ SMAD (22)) or a mutated 3'Sp1 site ($m3'Sp1$; Fig. 1). CTGF promoter/reporter constructs (0.5 μ g/well) alone with expression vector (as indicated; 1 μ g/well) were transfected into fibroblasts cultured from dermal scleroderma lesions. The construct containing a mutation in the 3'Sp1 site possesses 50% less activity than the full-length, wild-type construct. Administration of 150 nM mithramycin also caused a reduction in CTGF promoter activity.

wild-type CTGF promoter/reporter construct or with an otherwise identical construct with a mutation in its functional 3'Sp1 binding site (Fig. 3). Mutation of the 3'Sp1 site resulted in a 50% decrease of basal CTGF promoter activity in SSc fibroblasts (Fig. 3). Similarly, incubation of cells with the Sp1-specific inhibitor mithramycin (26) reduced elevated CTGF promoter activity (Fig. 3) and protein expression (Fig. 4). Mithramycin also blocked the TGF β -mediated induction of CTGF protein in normal fibroblasts, reflecting the fact that Sp1 is essential for basal CTGF promoter activity in fibroblasts. Collectively, our results suggest that the constitutive overexpression of CTGF in SSc fibroblasts was Sp1-dependent.

Elevated Levels of Sp1 Binding Exist in Nuclear Extracts from SSc Fibroblasts—We then assessed whether the requirement for Sp1 for the elevated levels of CTGF observed in SSc fibroblasts was mirrored by an elevated level of Sp1 binding to its binding site. We addressed this issue by evaluating the total level of cellular Sp1 capable of binding an Sp1 binding element. We compared nuclear protein from fibroblasts from four healthy subjects with fibroblasts with five subjects with SSc using two related methods. First, we used an enzyme-linked immunosorbent assay that measures Sp1 binding to an Sp1 response element (Fig. 5A). Second, we used a direct gel-shift assay with the same Sp1 binding sequence as probe for consistency. According to either assay, subjects with SSc possessed significantly elevated levels of Sp1 binding relative to healthy subjects (Fig. 5B). As a control, we pooled our normal and SSc nuclear extracts and hybridized equal proteins amounts to a gel-shift microarray, which contained double-stranded oligomers bearing consensus DNA binding sites of known transcription factors. We found that, in contrast to our results examining Sp1 binding, nuclear extracts from normal and SSc fibroblasts contained equal amounts of binding to a consensus oligomer containing a binding site for the nuclear factor of activated T cells (Fig. 5C). To assess whether phosphorylation of Sp1 played a role in the Sp1 binding in fibroblasts, we preincubated nuclear extracts from normal and SSc fibroblasts with calf intestinal alkaline phosphatase before use in EMSAs. We found that removal of phosphates enhanced binding of Sp1 to DNA, suggesting that in fibroblasts, phosphorylation of Sp1 normally suppresses Sp1 binding to DNA (Fig. 5D). Thus, our

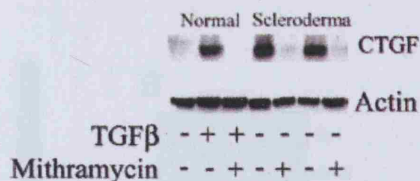


Fig. 4. Effect of mithramycin on CTGF expression in dermal fibroblasts. After culturing cells to 85% confluence, media were changed, and the Sp1 inhibitor mithramycin (150 nM) was added for 24 h to normal dermal fibroblasts, in the presence or absence of TGF β 1 (2.5 ng/ml), and to fibroblasts cultured from dermal lesions of patients with diffuse scleroderma. Cell layers were then lysed and equal amounts of protein (25 μ g) were subjected to SDS/PAGE and Western blot analysis with an anti-CTGF antibody. Addition of mithramycin blocks the basal CTGF expression in scleroderma fibroblasts. Blots were also probed with an anti-actin antibody to establish that lanes were equally loaded.

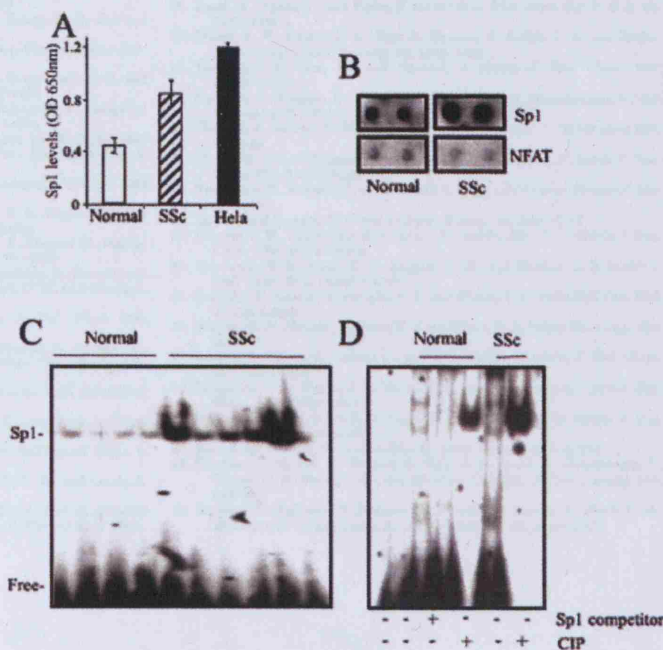
results suggest that the elevated level of CTGF expression observed in SSc fibroblasts is dependent, at least in part, on an elevated level of Sp1 binding present in the nuclei of SSc fibroblasts and is relatively independent of sequences involved with the TGF β induction of CTGF. These results are consistent with the notion that the fibrotic phenotype of the SSc fibroblasts is not caused solely by hyperactive or autocrine TGF β signaling.

DISCUSSION

Previously, we identified the CTGF promoter's TGF β response element, which is both necessary and sufficient to confer TGF β responsiveness to a heterologous promoter (22–24). However, this TGF β response element is dispensable for the elevated, constitutive level of CTGF expression that is the hallmark of the fibrotic phenotype of the SSc fibroblast; removal of this element has no significant impact on the elevated level of CTGF promoter activity in SSc fibroblasts (22, current study). Here, we have found that Sp1 is involved in CTGF promoter activity. Targeting Sp1 markedly reduced CTGF expression in SSc fibroblasts. Furthermore, nuclear extracts from SSc fibroblasts possessed significantly elevated levels of Sp1 binding to DNA. These results suggest that targeting mechanisms involved with Sp1 or its activation may provide useful in developing antifibrotic therapies.

Our results are intriguing in light of recent observations concerning the role of Sp1 in matrix gene regulation. Functional Sp1 binding motifs have been found in the minimal promoters of several collagen genes. For example, mithramycin inhibits collagen α 1 type I protein expression in normal fibroblasts (28), and Sp1 has been shown to activate expression of this gene's promoter (29). Furthermore, analysis of the collagen α 2 type I promoter has shown that TGF β activates its expression through a complex process involving SMAD3 and -4 and Sp1 (30). In addition, Sp1 was shown to directly contribute to the expression of a wide variety of matrix genes (31). However, none of these studies examined the role of Sp1 and in the regulation of fibrogenic genes in a fibrotic setting. Intriguingly, Sp1 or its family members may contribute directly to the elevated level of expression of target genes in fibrosis. Intriguingly, increased phosphorylation of Sp1 has been shown to be a feature of scleroderma fibroblasts (32). However, the relevance of the phosphorylated residues to Sp1 binding or the elevation of matrix gene expression in SSc fibroblasts is not clear. In this study, we found that phosphorylation of Sp1 had little effect on the ability of Sp1 to bind to its consensus binding element; addition of alkaline phosphatase to nuclear extracts before performing a gel shift with an oligomer bearing a consensus

FIG. 5. Elevated levels of Sp1 binding to DNA exists in nuclear extracts from fibroblasts cultured from dermal lesions of SSc patients. A, an enzyme-linked immunosorbent assay, which detects binding of Sp1 or Sp1-like binding activity to a double-stranded oligonucleotide bearing a consensus Sp1 binding motif, was used to detect relative levels of Sp1 binding activities in nuclear extracts prepared from dermal fibroblasts cultured from four healthy subjects as well as five subjects with diffuse SSc (mean \pm S.D., $n = 12$). B, a gel shift using a double-stranded oligonucleotide identical to that used in A. C, a microarray containing double-stranded oligomer bearing consensus binding elements of known transcription factors was probed using nuclear extracts prepared from healthy subjects and subjects with diffuse SSc. Elevated binding of nuclear factors to a consensus Sp1 binding element, but not a consensus nuclear factor of activated T cells (NFAT) binding element, is shown. D, phosphorylation of Sp1 or Sp1-like binding activity suppresses Sp1 binding to DNA. Nuclear extracts from healthy subjects and subjects with SSc were pretreated for 1 h at 37 °C with calf intestinal alkaline phosphatase (CIP) before performing an electrophoretic mobility shift assay with a consensus Sp1 binding site. Dephosphorylation of extracts eliminates the faster mobility DNA/protein complexes, indicating that these complexes represent the phosphorylated form of Sp1 binding to DNA. However, the overall binding of Sp1 to its DNA binding site increases.



Sp1 element enhanced Sp1 binding to DNA. Our results are, in fact, consistent with data from other systems showing that Sp1 binding is repressed by phosphorylation and that removal of phosphate groups from Sp1 enhances binding to DNA (33–39). However, it is possible that phosphorylation of Sp1 or Sp1-like proteins could enhance the ability of Sp1 to form an active transcriptional complex with other nuclear factors. That said, our report is the first to directly assess the potential, functional contribution of Sp1 to the fibrotic phenotype of the SSc fibroblast.

The elevated level of Sp1 binding observed in our studies could arise from an increased amounts of Sp1 protein in SSc nuclear extracts compared with extracts prepared from healthy subjects. However, when we directly addressed this issue by using Western blot analysis of nuclear extracts with an anti-Sp1 antibody, we could show no appreciable differences in Sp1 protein levels between nuclear extracts prepared from healthy subjects and those prepared from persons afflicted with SSc (data not shown). Sp1 is a member of the Kruppel-like family of proteins that effectively bind to a consensus Sp1 binding site (40). Thus, it is possible that the elevated Sp1 binding activity observed in SSc nuclear extracts could be caused by other members of the Kruppel-like Sp1 family and that these members show elevated expression in lesional diffuse SSc fibroblasts relative to normal fibroblasts. In fact, we have shown that other Kruppel-like family members are capable of activating the CTGF promoter (data not shown). The precise identity of the proteins binding to the Sp1 binding site in the CTGF promoter, and whether expression of these proteins is elevated in SSc, is currently under investigation.

In conclusion, by investigating the constitutive CTGF expression from defined fibrotic settings, we have shown that the

TGF β response element in the CTGF promoter is dispensable for its constitutive up-regulation in SSc fibroblasts; rather, it seems to reflect an elevation of basal promoter activity as visualized by its dependence on Sp1. Sp1 generally acts with other transcription factors to potentiate transcription (for review, see Ref. 40), so it is likely that Sp1 acts with these factors to activate transcription of genes, such as CTGF, expressed in SSc fibroblasts. Identification of these additional factors is currently underway. That said, our results suggest that a simple model of autocrine TGF β signaling seems to be insufficient to explain the fibrotic phenotype of the SSc fibroblast. For example, prolonged exposure of fibroblasts to TGF β is insufficient to generate fibroblasts that constitutively overexpress collagen relative to their normal counterparts (8). Because CTGF has been reported to be an effective marker of fibrosis, and given the known profibrotic effects of this molecule and that small molecule inhibitors that suppress CTGF expression alleviate fibrotic effects *in vivo* and *in vitro* (41, 42), further identification of key transcription factors and signaling pathways involved in regulating CTGF promoter activity in SSc is highly likely to have a significant impact on the development of novel therapeutic agents that could combat this debilitating disorder.

REFERENCES

- Gruschwitz, M., Muller, P. U., Sepp, N., Hofer, E., Fontana, A., and Wick, G. (1990) *J. Invest. Dermatol.* 94, 197–203.
- Peltonen, J., Kahari, L., Uitto, J., and Jimenez, S. A. (1990) *J. Invest. Dermatol.* 94, 365–371.
- Blobe, G. C., Schiemann, W. P., and Lodish, H. F. (2000) *N. Engl. J. Med.* 342, 1350–1358.
- Cotton, S. A., Herrick, A. L., Jayson, M. I., and Freemont, A. J. (1998) *J. Pathol.* 184, 4–6.
- Querfeld, C., Eccles, B., Huelskamp, C., Kreig, T., and Sollberg, S. J. (1999) *J. Dermatol. Sci.* 21, 18–22.

6. Kikuchi, K., Hard, C. W., Smith, E. A., LeRoy, E. C., and Trojanowski, M. (1992) *Biochem. Biophys. Res. Commun.* 187, 45-50.
7. Morland, L. W., Goldsmith, K. T., Russell, W. J., Young, K. R. Jr., and Currier, R. I., Jr. (1992) *Ann. J. Med.* 93, 528-536.
8. McWhirter, A., Colucci, P., Babin, K., Miyasaka, K., and Black, C. (1994) *Lab. Invest.* 71, 606-604.
9. Iguchi, A., Nishino, K., Kikuchi, K., Sato, S., Ima, H., Grotendorst, G. R., and Takahara, K. (1995) *J. Invest. Dermatol.* 105, 290-294.
10. Sen, S., Nagata, T., Hasegawa, M., Tamotani, T., Nakashiki, T., Takigawa, M., and Takahara, K. (2000) *J. Rheumatol.* 27, 149-154.
11. Mossad, E. K. A., and Brigstock, D. A. (2000) *Mol. Genet. Metab.* 71, 276-292.
12. Leach, A., Holmes, A., and Abraham, D. J. (2002) *Curr. Rheumatol. Rep.* 4, 136-142.
13. Bradham, D. M., Iguchi, A., Potter, R. L., and Grotendorst, G. (1991) *J. Cell Biol.* 114, 1285-1294.
14. Brigstock, D. R., Stiffen, C. L., Kim, G. Y., Vegueta, R. K., Diehl, J. R., and Harding, P. A. (1997) *J. Biol. Chem.* 272, 20275-20282.
15. Duncan, M. R., Framer, K. S., Abramson, S., Williams, S., Klapper, H., Huang, X., and Grotendorst, G. R. (1999) *FASEB J.* 13, 1774-1786.
16. Shi-wen, X., Pennington, D., Holmes, A., Leach, A., Bradham, D., Benuechamp, J. R., Fonseca, C., du Bois, R. M., Martin, G. R., Black, C. M., and Abraham, D. J. (2000) *Exp. Cell Res.* 259, 215-224.
17. Iguchi, Y., Truter, S., and Ramirez, F. (1994) *J. Biol. Chem.* 269, 14828-14834.
18. Mori, T., Kikuchi, M., Shinonaka, N., Hayashi, T., Kobayashi, A., Iguchi, M., Takigawa, T., Nakashiki, T., and Takahara, K. (1999) *J. Cell Physiol.* 181, 153-159.
19. Abreu, J. G., Katsura, N. I., Revuevade, B., and De Robertis, E. M. (2002) *Nat. Cell Biol.* 4, 599-604.
20. Abraham, D. J., Shiwen, X., Black, C. M., Su, S., Xu, Y., and Leach, A. (2000) *J. Biol. Chem.* 275, 15220-15225.
21. Grotendorst, G. R., Okochi, H., and Hayashi, N. (1996) *Cell Growth Differ.* 7, 499-499.
22. Holmes, A., Abraham, D. J., Su, S., Shiwen, X., Black, C. M., and Leach, A. (2001) *J. Biol. Chem.* 276, 10594-10601.
23. Blom, I. E., Goldschmidt, R., and Leach, A. (2002) *Mov. Disord.* 17, 473-482.
24. Leach, A., Holmes, A., Black, C. M., and Abraham, D. J. (2002) *J. Biol. Chem.* 277, 3008-3015.
25. Leach, A., Byrne, C., and Fuchs, E. (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88, 7945-7952.
26. Hume, S. W., Snyder, R. C., Roy, R., Thomas, S., Koller, C. A., and Miller, D. M. (1991) *J. Clin. Invest.* 88, 1612-1621.
27. Vercellia, F., Chu, M., and Mauviel, A. (2001) *J. Biol. Chem.* 276, 17059-17063.
28. Nohle, M. C., Brunner, D. A., Grun, H. J., Dierbach, H., Martelmann, R., and Herrmann, F. (1998) *J. Clin. Invest.* 92, 2916-2921.
29. Chen, S. J., Artlett, C. M., Jimenez, S. A., and Varga, J. (1998) *Gene* 215, 101-110.
30. Zhang, W., Ou, J., Iguchi, Y., Greenwel, P., and Ramirez, F. (2000) *J. Biol. Chem.* 275, 39267-39269.
31. Vercellia, F., Rosset, J., and Mauviel, A. (2001) *J. Invest. Dermatol.* 116, 785-789.
32. Ima, H., and Tanaka, K. (2000) *Arthritis Rheum.* 43, 2240-2247.
33. Leggett, R. W., Armstrong, S. A., Barry, D., and Mueller, C. R. (1995) *J. Biol. Chem.* 270, 25979-25984.
34. Armstrong, S. A., Barry, D. A., Leggett, R. W., and Mueller, C. R. (1997) *J. Biol. Chem.* 272, 13490-13495.
35. Borek, F., Aquino, A., Joseph, S. F., and Glasser, R. I. (1990) *Mol. Cell Biol.* 10, 5541-5547.
36. Watkins, R. D., Newrot, T., Potts, R. J., and Hart, B. A. (2003) *Toxicology* 184, 157-170.
37. Lacoux, I., Lipsey, C., Imbert, J., and Kahn-Pollak, B. (2002) *J. Biol. Chem.* 277, 9506-9508.
38. Martenson, E. R., Marks, P. A., Shiozaki, A., and Marchant, J. L. (1997) *J. Biol. Chem.* 272, 10540-10547.
39. Daniel, S., Zhang, S., DePaulis-Bosch, A. A., and Kim, K. H. (1996) *J. Biol. Chem.* 271, 14892-14897.
40. Kaczynski, J., Cook, T., and Urvita, R. (2008) *Genome Biol.* 4, 306.
41. Stratton, R., Shiwen, X., Martini, G., Holmes, A., Leach, A., Haberbauer, T., Martin, G. R., Black, C. M., and Abraham, D. (2001) *J. Clin. Invest.* 108, 241-250.
42. Stratton, R., Rajkumar, V., Postico, M., Nichols, B., Shiwen, X., Black, C. M., Abraham, D. J., and Leach, A. (2002) *FASEB J.* 16, 1949-1951.

New insights into the role and regulation of CTGF/CCN2 in fibrotic disorders

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Abstract

The CCN (*cyr61*, *CCN2*, *nov*) family of modular proteins regulate diverse biological affects including cell adhesion, matrix production, tissue remodelling, proliferation and differentiation. Recent targeted gene disruption studies have demonstrated the CCN family to be developmentally essential for chrondrogenesis, osteogenesis and angiogenesis. A large body of *in vivo* and *in vitro* data implicates this family of genes in a host of pathological conditions including vascular and fibrogenic diseases such as cancer and scleroderma. Given this, the use of this family of genes as potential prognostic markers and therapeutic targets in fibrotic diseases has been postulated. This review will focus on perhaps the best studied member of this modular matricellular protein family, CCN2, its structure, regulation, biological actions and the recent progress in understanding its function in normal and pathological situations and the implications for the future anti-fibrotic therapies.